Molecular and phenotypic characterization of *Candida albicans* bloodstream isolates from a German university hospital

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Index of Contents

List of abbreviations

ANOVA	analysis of variance: statistical tool, amongst other functions used for phylogenetic interpretations of clade-specific properties
ATP	adenosine triphosphate: coenzyme essential for intracellular energy transfer
C.	abbreviation for the genus *Candida*
CABI	*Candida albicans* bloodstream isolate
CBP	clinical breakpoint: interpretation of the relevance of an antifungal MIC for clinical therapy
Cdr1, Cdr2	Candida drug resistance transporter: Multidrug transporter; transport phospholipids in an in-to-out direction
CFU	colony forming units (used to inoculate agar medium dishes)
CLSI	Clinical and laboratory standard institute
DST	diploid sequence type: defined by an MLST-profile of 7 gene fragments
ECOFF/ECV	epidemiological cutoff value: splitting populations in wild-type and non-wild-type due to their statistical MIC distribution
Erg3p	C-5 sterol desaturase: catalytic protein of the *Candida* ergosterol biosynthesis pathway
Erg11p	lanosterol-14α-demethylase: catalytic protein of the *Candida* ergosterol biosynthesis pathway
EUCAST	European committee on antimicrobial susceptibility testing
ITS	internal transcribed spacer region: DNA sequence that is used to identify *Candida* species
MALDI-TOF-MS	matrix assisted laser desorption ionization - time of flight - mass spectrometry: method that can be used to identify *Candida* species
Mdr	multidrug resistance transporter: efflux pump for multiple drug substrates
MEGA	molecular evolutionary genetics analysis: software to create phylogenetic trees
MIC	minimal inhibitory concentration in µg/ml: specifies antifungal drug concentration that is needed in order to significantly inhibit the *in vitro* growth of microbes
ML	Maximum Likelihood: algorithm used to create phylogenetic trees
MLST	multilocus sequence typing: sequence-based method to characterize distinct isolates of the same species

NRZMyk	German National Reference Center for Invasive Fungal Infections
PCR	polymerase chain reaction: method to amplify small DNA-sequences *in vitro*
RPMI agar	culture media designed specifically for gradient-dependent antifungal susceptibility tests
SNP	single nucleotide polymorphism: variable site in an otherwise conserved DNA sequence
ST	sequence type: used to describe sequence variants of MLST-relevant gene loci, also called allele (refers only to one locus, a combination of sequence types from several loci yields a DST)
UPGMA	unweighted pair group method wit arithmetic mean: algorithm used to create phylogenetic trees
YPD	yeast extract peptone dextrose medium: culture medium for *Candida*, used either as liquid broth or solid agar

Genes:

AAT1 - aspartate amino transferase, housekeeping gene used for MLST analysis

ACC1 - acetyl-coenzyme A carboxylase, housekeeping gene used for MLST analysis

ADP1 - ATP-dependent permease, housekeeping gene used for MLST analysis

ERG3 - C-5 sterol desaturase: gene of the *Candida* ergosterol biosynthesis pathway

ERG11 - lanosterol-14α-demethylase: gene of the *Candida* ergosterol biosynthesis pathway

FKS - essential β-1,3-glucan synthase subunit, gene that plays an important role in echinocandin resistance

MAT1α - regulator of the α mating type; gene of the MTLα (Mating Type Like) locus: determines the mating type of a *Candida* isolate

MPI - mannose phosphate isomerase, housekeeping gene used for MLST analysis

SYA1 - alanyl-RNA synthetase, housekeeping gene used for MLST analysis

UPC2 - zinc finger transcription factor, regulator of genes from the ergosterol biosynthesis pathway in *Candida*

VPS13 - vacuolar sorting protein, housekeeping gene used for MLST analysis

ZWF1 - glucose-6-phosphate dehydrogenase , housekeeping gene used for MLST analysis

1 Zusammenfassung

Bakterielle Infektionen sind nach wie vor die Hauptursache für Sepsis. Seit den neunziger Jahren hat jedoch die Zahl der Pilzinfektionen erheblich zugenommen. Systemische fungale Infektionen sind als Krankheitsursache gerade deshalb interessant, weil die Erreger meist endogene Kommensalen des Wirts sind. *Candida*-Spezies sind ein typisches Beispiel für einen solchen Infektionsverlauf. Von Bedeutung sind vor allem *Candida albicans* und *Candida glabrata*, sie werden im Schnitt weltweit in etwa 5-20% aller Blutkulturen nachgewiesen.

Diese Dissertation beschäftigt sich mit *Candida*-Isolaten, die zwischen 2005 und 2012 aus Blutkulturen von Patienten des Universitätsklinikums Würzburg kultiviert wurden. Im Rahmen des Projektes erfolgte eine detaillierte phänotypische und molekulare Charakterisierung der 99 Isolate. Die *Candida*-Isolate wurden hinsichtlich Spezieszugehörigkeit, individueller genetischer Variabilität und Suszeptibilität gegenüber üblichen antifungalen Wirkstoffen untersucht. Die resultierende individuelle Charakterisierung jedes Isolats wurde für weiterführende epidemiologische und phylogenetische Analysen genutzt.

Distinktive und untereinander vergleichbare genotypische Profile der isolierten *Candida albicans* – Stämme wurden mit Hilfe von Multilocus Sequenztypisierung (MLST) erstellt. Basiert auf Einzelnukleotidpolymorphismen in konservierten DNA-Abschnitten werden den Isolaten individuelle diploide Sequenztypen zugeordnet. Diese Sequenzprofile können mit Hilfe einer internationalen Datenbank verglichen und zu phylogenetischen Analysen herangezogen werden können.

Bei Isolaten, die während der MLST-Analyse experimentelle Probleme verursachten, wurde die Spezieszugehörigkeit überprüft. Dazu wurden Hyphenformation in Flüssigkultur und Wachstumsphänotyp auf CHROM-Agar untersucht. In einigen Fällen wurde zur Absicherung eine ITS-PCR (Amplifikation eines speziestypischen DNA-Bereichs) durchgeführt.

Die genotypische Feintypisierung ergab für 95 Isolate ein individuelles MLST-Profil, welches zur Online-Datenbank hinzugefügt werden konnte. Dabei wies die Mehrheit der *C. albicans* Isolate (75 Stämme) einen zuvor noch nicht beschriebenen Sequenztyp auf. Drei Isolate wurden als *C. dubliniensis* identifiziert und von weiteren Analysen ausgeschlossen. Zwei Proben wurden als Mischkulturen aus *C. albicans* und *C. glabrata* erkannt, wobei es nur bei einem Isolat möglich war, die *C. albicans*-Komponente zu isolieren und zu analysieren.

Eine auf den MLST-Profilen basierte phylogenetische Analyse der *C. albicans* Isolate mit Hilfe des eBurst-Algorithmus ergab keinen Hinweis auf eindeutige phylogenetische Korrelationen. Trotz der engen geographischen Limitation und ihres klinisch ähnlichen Ursprungs (Blutkultur) wiesen die Isolate eine große genetische Varianz auf.

Bei *C. albicans* Isolaten mit identischen Sequenztypen wurden im Hinblick auf nosokomiale Transmission die entsprechenden Patientendaten eingesehen. Zum einen wurde somit die Konstanz des Sequenztypen bei subsequenten Isolaten von einem Patienten bestätigt, zum anderen wurde eine Sequenztyp-Gleichheit bei Isolaten von neugeborenen Zwillingen auf der Neonatal-Station festgestellt. In allen anderen Fällen bestand keine nachweisbare räumliche oder zeitliche Verbindung zwischen Patienten, bei denen genetisch weitgehend identische *C. albicans* Stämme in der Blutkultur auftraten, die Wiederholung des Sequenztyps wurde deshalb als rein zufällig angesehen.

Resistenztests für die antifungalen Wirkstoffe Amphotericin B, Caspofungin, Fluconazol, Itraconazol, Posaconazol und Voriconazol wurden nach standardisierten E-Test-Richtlinien durchgeführt. Unter den 95 getesteten Isolaten wurden größtenteils keine signifikanten Resistenzen gegen die genannten Wirkstoffe festgestellt.

Insgesamt konnten im Verlauf dieses Dissertationsprojektes die analysierten *Candida*-Blutkulturisolate durch MLST-Profile und Suszeptibilitätstests detailliert charakterisiert werden. Die Ergebnisse der genotypischen Analyse zeigen den hohen Stellenwert der MLST bei der Nachverfolgung nosokomialer Transmissionsrouten und bei epidemiologischen Betrachtungen. Die erhaltenen Daten stellen eine wichtige Grundlage für weitere Experimente mit den klinischen Isolaten dar.

2 Introduction

Next to a variety of bacteria and archeae, fungi are a common part of the normal microbial flora on human mucosal surfaces (Rajilic-Stojanovic und de Vos 2014). Recent studies of the resident microbiota in the human gastrointestinal tract revealed that as much as 66 fungal genera can be present in healthy individuals (Mukherjee et al. 2015). The majority of those intestinal fungi are yeasts, *Candida spp*. being the most prevalent (Rajilic-Stojanovic und de Vos 2014). *Candida* species of clinical significance as commensals and opportunistic pathogens of the human host are *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. dubliniensis* and *C. rugosa* (Rajilic-Stojanovic und de Vos 2014, McManus und Coleman 2014). *C. albicans* is the predominant and clinically most relevant of the mentioned species (McManus und Coleman 2014). As the major cause of systemic candidiasis, it is regarded as the most pathogenic *Candida* species that colonizes human hosts (Moran et al. 2004). One explanation for its frequent role as a pathogen might be its high prevalence. *C. albicans* is established in the gastrointestinal mycobiota very early. The species can be detected in over 95% of one-month-old infants, illustrating how commonly it colonizes the body without causing diseases (Kumamoto und Vinces 2005). Yet as soon as opportunity arises, the harmless commensal is able to act as a pathogen. If the host's immune system is compromised, the indigenous *C. albicans* population can cause infections ranging from a superficial mucosal thrush to an invasive, life-threatening candidiasis (Kumamoto und Vinces 2005). Its success as a pathogen is strongly supported by the polymorphic phenotype of *C. albicans*, which grows as yeast as well as in a filamentous form (McManus und Coleman 2014). As one of only two *Candida* species that is capable of forming true hyphae (figure 1), *C. albicans* has considerable advantage during adhesion and tissue invasion, crucial processes during infection (Kumamoto und Vinces 2005, Stokes et al. 2007).

Dissemination of *Candida* in the bloodstream, known as invasive candidiasis or candidemia, is a life-threatening condition. The frequency of nosocomial systemic *Candida* infections has been increased significantly during the last decades (Colombo et al. 2014, Mikulska et al. 2012). Candidemia was diagnosed with an incidence of 6.9/1000 patients in ICUs worldwide (Kett et al. 2011), which appears to be relatively low. Nevertheless, Wisplinhoff et al found *Candida spp.* to be the fourth leading cause (9%) of bloodstream infections in hospitalized patients in the US (Wisplinghoff et al. 2004). Interestingly, those *Candida*-associated bloodstream infections showed the highest crude mortality (39.2%) in the mentioned study (Wisplinghoff et al. 2004). Systemic candidiasis is frequently fatal, especially if the infection causes a systemic inflammatory response syndrome or sepsis. The mortality rate of

nosocomial candidemia is generally estimated between 30-60% (Kett et al. 2011, Bougnoux et al. 2008, Gudlaugsson et al. 2003). At risk for a disseminated *Candida* infection are all hospitalized patients with considerable immune deficiencies. Typical risk factors among ICU patients are long-term antibiotic treatment, solid tumors, cancer chemotherapy, persisting intravascular catheters and organ transplantation attended by medical immune suppression (Giri und Kindo 2012). Candidemia is not a problem exclusively encountered in the ICU; it is also a condition that affects multi-morbid patients in internal medicine wards with increasing frequency (Mikulska et al. 2012). Old age, parental nutrition and bacterial infections are typical comorbidities in this group (Bassetti et al. 2011).

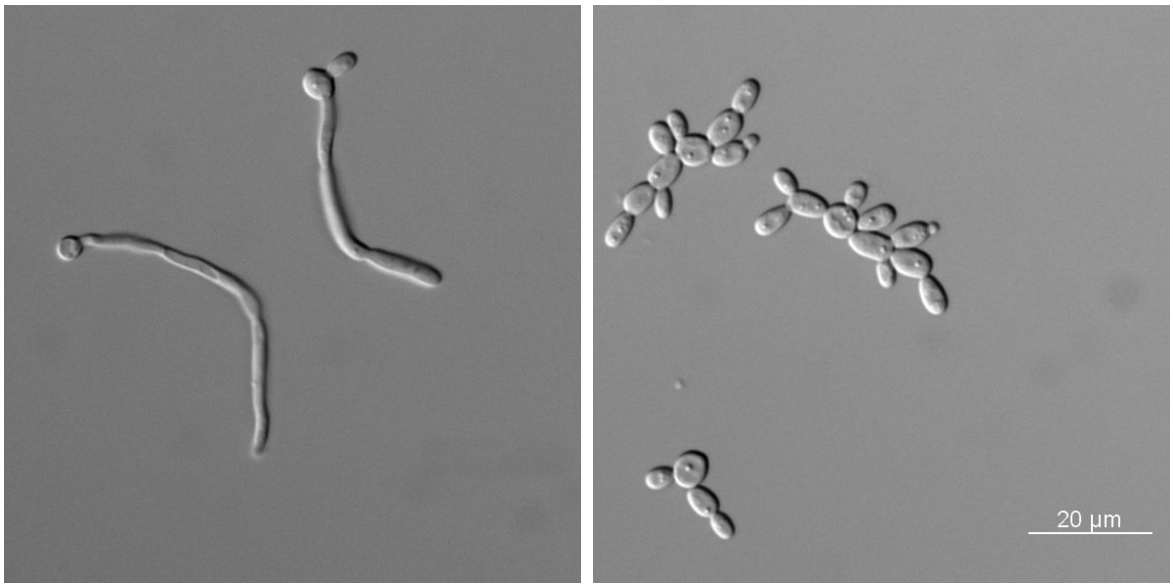

Figure 1: *Candida albicans* phenotype. Filamentous (hyphae) and planctonic (yeast) growth mode of *C. albicans*. The transition facilitates adhesion and tissue invasion during a systemic infection and is considered to be one of the central pathogenity mechanism of *C. albicans* . Microscopic images were taken at the ZIK Septomics facility Jena.

Despite continuous improvements of diagnostic methods, prophylaxis and therapy timing, successful treatment of candidemia remains challenging (Mikulska et al. 2012). Although the proportion of bloodstream infections caused by other, sometimes intrinsically resistant *Candida* species increased recently, *C. albicans* still remains the leading cause of fungemia (Mikulska et al. 2012, Kett et al. 2011). Next to clinical efforts to decrease candidemia mortality, lab-side analysis of isolated *C. albicans* strains is important to understand epidemiology, nosocomial transmission routes and development of resistances (Odds und Jacobsen 2008).

With the increasing incidence of nosocomial candidemia episodes, several efforts of molecular strain typing were made during the last decades (Lyon et al. 2010). As a diploid

organism with a high degree of genome plasticity, *C. albicans* posed certain challenges to the accuracy of sequence-based typing methods (McManus und Coleman 2014). Clinical isolates were found to exhibit significant karyotype variability (Chibana et al. 2000), which might be owed to *C. albicans´* high tolerance of chromosomal rearrangements and even ploidy changes (McManus und Coleman 2014). The predominantly clonal reproduction cycle can be supplemented by a parasexual mating mechanism during times of environmental stress (Forche et al. 2009). The initially tetraploid offspring of this mating undergoes subsequent chromosome loss until the diploid state is reaccomplished (Bennett und Johnson 2003). The absence of a true meiosis process allows the high karyotypic variation observed in *C. albicans*, for major chromosomal rearrangements are not fatal for the offspring (Chibana et al. 2000). Even loss of heterozygosity and aneuploidy occur frequently and are tolerated well (McManus und Coleman 2014). This genomic plasticity enables *C. albicans* strains to undergo genetic microvariations during host colonization, infection and through strain transmission (Bougnoux et al. 2006).

Commensal strains are maintained by the host over long periods of time and are often the endogenous source of arising infections (McManus und Coleman 2014). Even so, it is important to employ suitable molecular typing systems to enhance the understanding of the population dynamics of the pathogen and to discover possible origins of nosocomial infections and drug-resistant strains (Lyon et al. 2010). A proper typing system facilitates the assignment of isolates to clades or clusters of a suspected founding genotype and its offspring, which is an essential tool for phylogenetic analysis (Lyon et al. 2010).

Before the widespread use of molecular typing techniques, efforts were made to classify *C. albicans* isolates phenotypically (McManus und Coleman 2014). But methods like serotyping, morphotyping and antimicrobial agent susceptibility testing lacked discriminatory power and reproducibility (McManus und Coleman 2014). Although multi-locus enzyme electrophoresis (MLEE) was a more reliable phenotypic typing method, it was not sufficiently able to detect small genetic variations (Saghrouni et al. 2013). Prior to the common availability and affordability of DNA sequencing, molecular typing methods targeted alterations in restriction endonuclease cleavage sites (McManus und Coleman 2014). The major disadvantage of DNA-based, non-sequencing typing methods like restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD) and electrophoretic karyotyping (EK) is that results are laboratory dependent (Bougnoux et al. 2002). A combination of restriction endonuclease digestion and southern blot hybridization, the Ca3 fingerprinting probe, is the non-sequence based typing method with the highest discriminatory power

(0.993) (Robles et al. 2004). This method is hold as reproducible and serviceable for computer-assisted phylogenetic analysis (Lyon et al. 2010). Isolates analyzed with Ca3 fingerprinting could be assigned to three distinct, geographically widespread clades that are still viable today (Robles et al. 2004). Two additional clades showed a geographical enrichment for Southern Africa and Europe (the SA and E clades) (McManus und Coleman 2014). Despite those promising features, Ca3 fingerprint analysis did not establish itself as the predominant molecular typing method, mainly because it is time-consuming, technically demanding and the results can not be easily compared or shared between different laboratories (McManus und Coleman 2014).

The gold standard of molecular typing is based on the direct analysis of DNA sequences. Multilocus sequence typing (MLST) relies on the PCR amplification and DNA sequencing of seven highly conserved DNA sections (Bougnoux et al. 2002). MLST fulfills all requirements for an effective molecular typing system (McManus und Coleman 2014). Its resolution is sufficiently high to discriminate closely related strains as individual genotypes and to recognize identical strains in a sample of isolates (Bougnoux et al. 2002). MLST has a discriminatory power of 0.999, thus having a better resolution than Ca3 fingerprinting (McManus und Coleman 2014). The major advantage of the molecular typing assay over the Ca3 fingerprinting system is the high reproducibility and easy conduction of MLST (Tavanti et al. 2003). Subjectivity in data interpretation and inter-laboratory differences are negligible, thereby yielding a data output that can be shared and compared between different labs (Bougnoux et al. 2004). A serviceable typing system should be able to reflect only evolutionary genomic changes and neglect unstable variations that are the result of a high genomic plasticity (McManus und Coleman 2014). MLST is applied to *C. albicans* loci that are under stabilizing selection pressure, consequently delivering results that meet the contemplated demands (Tavanti et al. 2003). MLST data are utilizable for software-assisted phylogenetic analysis and it is possible to estimate the genetic distance between related isolates employing the MLST results (Odds et al. 2007). The last specific advantage of MLST in regard to *C. albicans* is that the system is serviceable for permanently diploid organisms. In reviewing the sequencing chromatograms of an MLST-locus, determination and identification of different nucleotides in heterozygous positions is possible (Odds und Jacobsen 2008).

Multilocus-sequence typing of *C. albicans* is based on sequence variations in 400-500bp fragments of seven housekeeping loci. Bougnoux and Tavanti et al. proposed a set of primers for the amplification of the seven genes that achieved universal acceptance (table 1) (Bougnoux et al. 2002, Tavanti et al. 2003). Some of those specific housekeeping gene

regions were chosen because of their high divergence from corresponding regions in the *Saccharomyces cerevisiae* ortholog (Bougnoux et al. 2002). Several loci encode highly polymorphic proteins that were already used in multilocus enzyme electrophoresis typing assays (Tavanti et al. 2003). All seven loci contain multiple variable sites and thereby hold a high discriminatory power, but are evolutionary conserved and under stabilizing selection pressure at the same time (Bougnoux et al. 2002). The variable sites that are of interest for MLST are single nucleotide polymorphisms (SNPs). Each locus contains 7 (*ACC1*) to 17 (*VPS13*) SNPs (see table 1) (Bougnoux et al. 2003). Local heterozygosity is frequent in those gene fragments, so the MLST data often show two different nucleotides in one variable position. The PCR primers (see table 1) amplify both alleles from homologous chromosomes in case of heterozygosity (Tavanti et al. 2003). The sequence chromatogram then shows two overlapping peaks of equal height, indicating the presence of both nucleotides in this diploid variable position (Odds und Jacobsen 2008).

The individual combination of nucleotides in the variable positions of one locus is defined as a genotype (the term "allele" is commonly used, too) (Bougnoux et al. 2002). All heterozygous variable positions are taken into account during the sequence analysis (Odds und Jacobsen 2008). Each genotype is numbered in the order of identification (Bougnoux et al. 2002).

Table 1. Summary of loci used for *C. albicans* MLST with current number of genotypes found for each locus (Tavanti et al. 2003, Bougnoux et al. 2003, http://calbicans.mlst.net/misc/info.asp, http://pubmlst.org/calbicans/)

Locus	Gene	Primer	seq. size (bp)	No. of SNPs	No. of geno-types
AAT1a	Aspartate Amino-transferase	Fwd 5'-ACTCAAGCTAGATTTTTGGC- 3' Rev 5'-CAGCAACATGATTAGCCC- 3'	349	10	159
ACC1	Acetyl-coenzyme A carboxylase	Fwd 5'-GCAAGAGAAATTTTAATTCAATG- 3' Rev 5'-TTCATCAACATCATCCAAGTG- 3'	407	7	106
ADP1	ATP-dependent permease	Fwd 5'-GAGCCAAGTATGAATGATTTG- 3' Rev 5'-TTGATCAACAAACCCGATAAT- 3'	443	16	146
MPIb	Mannose phosphate	Fwd 5'-ACCAGAAATGGCCATTGC- 3'	375	11	155

	isomerase	Rev 5'-GCAGCCATGCATTCAATTAT- 3'			
SYA1	Alanyl-RNA synthetase	Fwd 5'-AGAAGAATTGTTGCTGTTACTG- 3' Rev 5'-GTTACCTTTACCACCAGCTTT- 3'	391	13	207
VPS13	vacuolar protein sorting protein	Fwd 5'-TCGTTGAGAGATATTCGACTT- 3' Rev 5'-ACGGATGGATCTCCAGTCC- 3'	403	17	280
ZWF1b	Glucose-6-phosphate dehydrogenase	Fwd 5'-GTTTCATTTGATCCTGAAGC- 3' Rev 5'-GCCATTGATAAGTACCTGGAT- 3'	491	9	267

The total of seven individual genotypes is then combined to a distinct diploid sequence type (DST), which obtains a number, too (Bougnoux et al. 2002). All genotypes and diploid sequence types are registered and curated in a publicly available online database (http://pubmlst.org/calbicans/). The allelic variations in those seven relatively small DNA fragments provide sufficient information to describe clinical *C. albicans* isolates with high discriminatory power (Bougnoux et al. 2002). Through the online database it is possible to compare origins, prevalence and phylogeny of isolates internationally (Bougnoux et al. 2004).

Phylogenetic trees are an expression of the reconstructed evolutionary history of an organism. Whilst it is possible for animals and plants to build phylogenetic trees using comparative morphology and physiology and the support of fossil records, this method is insufficient for microbes (Nei und Kumar 2000). A more timely approach to study evolutionary relationships, even of the smallest organisms, is the molecular phylogenetic comparison of DNA sequences (Nei und Kumar 2000). A phylogenetic tree is able to represent estimated relationships between any kind of homologous DNA or protein sequences and their hypothetical common ancestors (Hall 2013). Thereby the molecular phylogenetic approach can avoid controversial systematic classifications like species, genera and taxa assignments (Nei und Kumar 2000). Suitable software, such as MEGA and eBurst, facilitates sequence-based phylogenetic analysis. There are several widely-accepted methods to estimate a phylogenetic tree from molecular data, for example Neighbor Joining, UPGMA/Maximum Parsimony, Bayesian Interference and Maximum Likelihood (ML) (Hall 2013). They are all based on the analysis of aligned homologous sequences; in case of *C. albicans* MLST analysis, the sequences of the

seven loci are concatenated before the alignment (Odds et al. 2007). Data analysis with MEGA or a similar software results in a dendrogram, whose resolution and significance are dependent on the amount of evolutionary caused sequence variation. For conserved DNA sections from different organisms belonging to the same species, an UPGMA or ML tree estimation is possible but can be seen controversial (Spratt et al. 2004). In case of *C. albicans* MLST data, the creation of a UPGMA dendrogram can be used to assign groups of related isolates to clades (Odds et al. 2007). Since all analyzed sequences differ only in defined SNP-positions and variation is frequently caused by recombination rather than mutation, the resulting tree is often incongruent (Planet 2006, Feil et al. 2004).

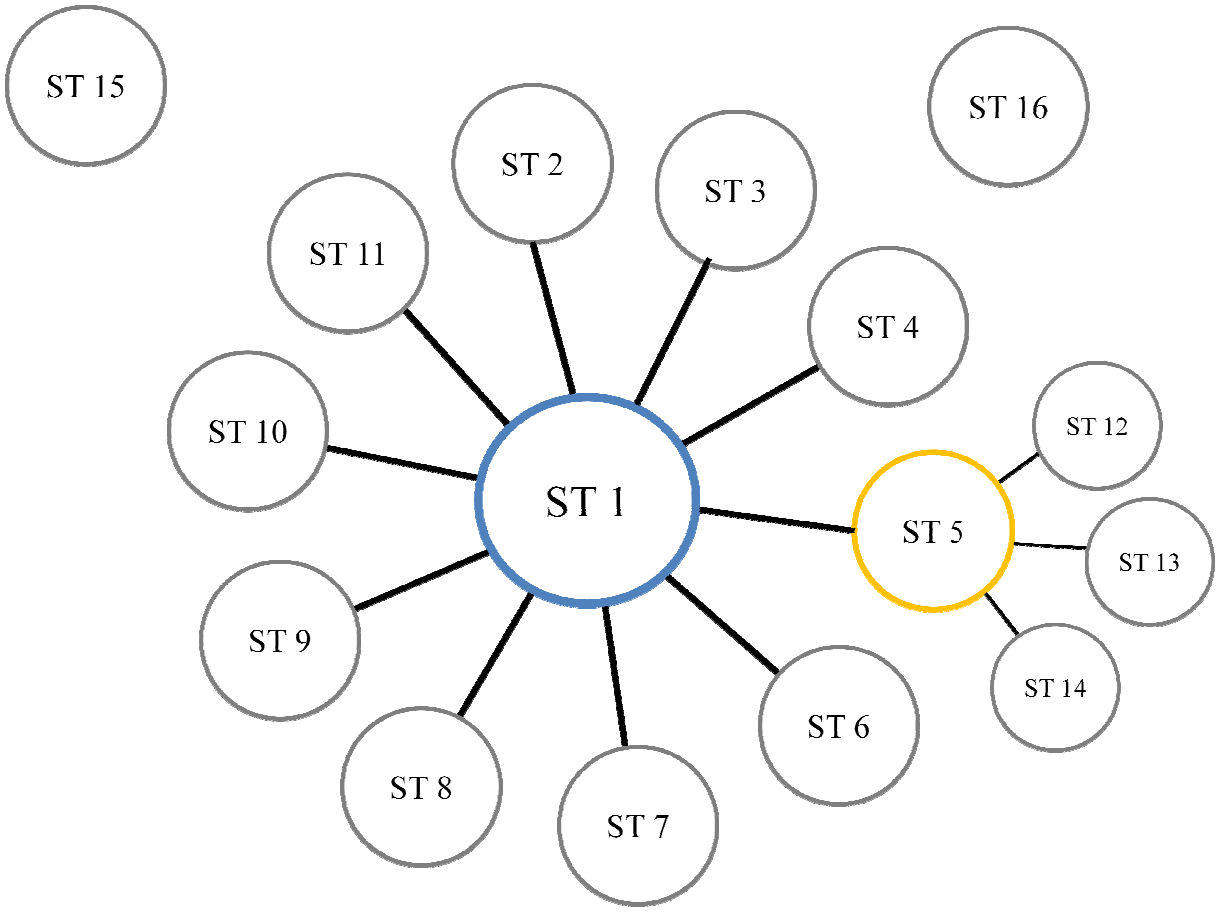

Figure 2: Schematic eBurst diagram. Highly similar sequence types (STs) are clustered in a radial pattern around a predicted founding ST (illustrated as ST1 in blue in this figure). Single locus variants (sharing 6 from 7 alleles with the founding ST) are connected with the founding ST by a direct link. Double locus variants (5/7 shared alleles) are connected indirectly with the founding ST if a subfounder (yellow, ST 5) was included in the analysis. If not, they are shown as singletons without connection to the founding ST (e.g. ST 15).

A more appropriate method to group highly similar sequence samples is the use of the eBurst algorithm (Figure 2) (Feil et al. 2004). The BURST method assumes that closely related isolates can be traced back to a founding genotype and clusters them in a so called “clonal

complex" around the predicted group founder (Figure 2) (Spratt et al. 2004). In application to MLST data, a clonal complex is formed from diploid sequence types that differ in only one or two alleles. The mutually exclusive groups are structured as a radial diagram with the predicted founding genotype in the center, expressing the hypothetical pattern of sequence diversification (Spratt et al. 2004).

Irrespective of the algorithm that is used to analyze MLST data, the formation of groups and clades is supposed to be relatively consistent (McManus und Coleman 2014). Odds et al. analyzed a large proportion of the *C. albicans* MLST database using MEGA and eBurst and defined 17 clades and 53 clonal clusters (Odds et al. 2007). The clades determined in a UPGMA dendrogram correlate well with clonal clusters from the eBurst analysis. Clonal clusters respect the clade boundaries, whereas clades can contain more than one clonal cluster (Odds und Jacobsen 2008). Clade 1 and respectively clonal cluster 1 are usually the largest specified groups of the phylogenetic analysis (Odds et al. 2007). The most common DST in this group is DST 69 (Odds et al. 2007). The clades 1-4 contain with ~67% of the analyzed DSTs the major proportion of the *C. albicans* isolates (Odds et al. 2007). Those clades fully correspond to the previously described clade assignments from Ca3 DNA fingerprinting, clades I, II, III and SA, respectively (Odds et al. 2007).

A univariate ANOVA was used to identify specific strain characteristics that are associated with clade designations in a statistically significant way (Odds et al. 2007). The analysis showed that only ABC type and geographical origin of the isolates were highly significant in relation to clade assignment (Odds et al. 2007). Mating type, time of isolation, anatomical origin and drug susceptibility did not show a significant correlation with the clade designation (Odds et al. 2007). Although a differential distribution of pathogenic and commensal isolates and of anatomical origins was observed to some extent, statistically significant clade distributions were not proven (McManus und Coleman 2014). Reduced susceptibility to azole antifungal agents was found in a low percentage of the isolates analyzed by Odds et al. (Odds et al. 2007). While the prevalence of those isolates in some clades (clades 5 and 6 and singletons) was subjectively higher than expected, there was no significant correlation between antifungal susceptibility and clade distribution (Odds et al. 2007). An enrichment of isolates with low flucytosine susceptibility in clade 1 was the only observation that could be proven to be statistically significant (Odds et al. 2007). These data correspond with former studies that assumed a restriction of genetic flucytosine resistance to Ca3 fingerprinting group I (Pujol et al. 2004). With respect to recombination events, blurring of geographical effects by

global travel and the influence of genes that are not considered in MLST analysis (Odds und Jacobsen 2008), all studies of clade-associated properties should be carefully reflected.

Resistance of *Candida spp.* to antifungal drugs poses an emerging problem in the treatment of patients with invasive fungal diseases, possibly leading to clinical failures (Maubon et al. 2014). Despite the availability of new and improved antifungal drugs, the mortality rates of patients with invasive candidiasis are still critically high (Alcazar-Fuoli und Mellado 2014). While the outcome of a serious bloodstream infection depends mainly on the status of the host, fast diagnosis of fungemia and a prompt antifungal treatment can have a major impact (Alcazar-Fuoli und Mellado 2014). The demand for an appropriate and speedy response to invasive fungal diseases constitutes a complex challenge. If the response of the clinical isolate to the chosen antifungal is poor, the result might be fatal (Pfaller 2012, Pfaller und Castanheira 2016). The seriousness of invasive candidiasis and the difficulties in prompt diagnosis increasingly lead to prophylactic and empirical use of antifungal drugs in high-risk patients and cases of persisting infections (Alcazar-Fuoli und Mellado 2014). The liberate application of antifungal therapy induces a problematic selective pressure in favor of antifungal resistances (Alcazar-Fuoli und Mellado 2014). Although resistance mechanism and their distribution are not as pronounced in fungi as in prokaryotes, insensitivity to antifungal drugs is a serious threat and should be a focus in the analysis of clinical isolates (Arendrup 2013, Lockhart et al. 2012).

The need for well-tolerated, host-compatible drugs limits the number of targets that can be affected by antifungal agents (Odds et al. 2003). Currently, three classes of antifungals are commonly used: triazoles, echinocandins and polyenes (Alcazar-Fuoli und Mellado 2014). Their action mechanisms are molecularly different, but usually interfere with the integrity of the fungal cell wall and membrane (Maubon et al. 2014). Additionally, there are some less popular antifungal agents like base analogues (e.g. flucytosine) and others available (Odds et al. 2003).

Triazoles like fluconazole, itraconazole and the newer broad-spectrum variants voriconazole and posaconazole are the largest class of antifungal agents (Odds et al. 2003, Maubon et al. 2014). Those very commonly applied drugs inhibit the synthesis of the main component of the fungal cell membrane, ergosterol (Terrell 1999). Targeting a catalytic protein from the ergosterol synthesis pathway, the lanosterol-14α-demethylase (also known as Cyp51p in *Aspergillus* spp. and Erg11p in *Candida* spp.), triazoles lead to ergosterol depletion in the fungal membrane (Figure 3) (Odds et al. 2003). Subsequently, membrane permeability and fluidity is altered and the activity of membrane-bound proteins is changed (Odds et al. 2003).

Erg11p inhibition also leads to accumulation of 14α-methylated sterols and the synthesis of unusual and toxic sterols by induction of Erg3p activity (Maubon et al. 2014).

Echinocandins, e.g. caspofungin, micafungin and anidulafungin, are a reliable and recommended treatment option for invasive candidiasis (Pfaller und Castanheira 2016) . They target proteins responsible for the synthesis of an important cell wall polysaccharide, (1,3)-β-D-glucan, thereby inhibiting the formation of a proper cell wall during cell growth (Figure 3) (Odds et al. 2003).

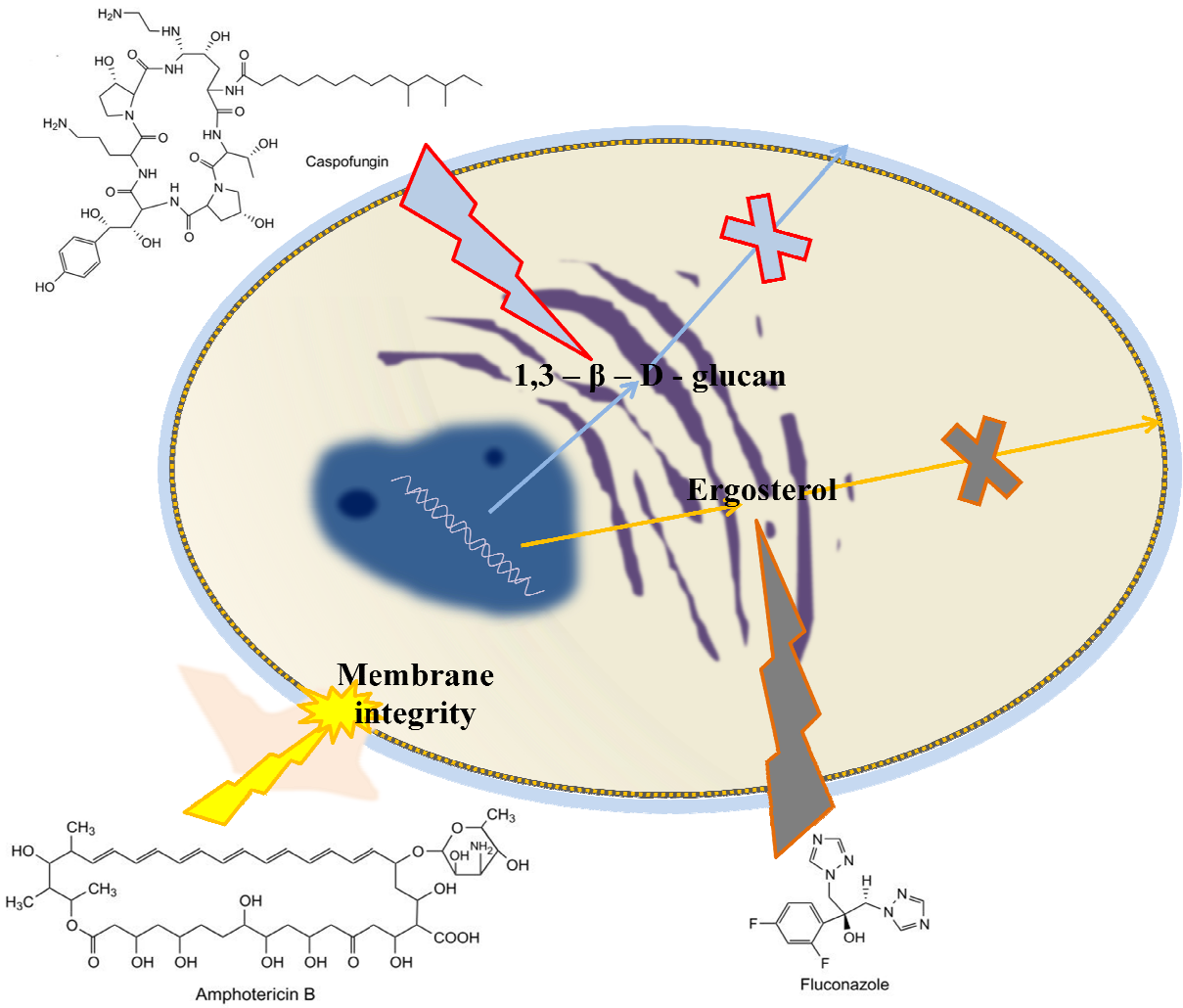

Figure 3: Effects of common antifungals on *Candida spp*. Triazoles, e.g. Fluconazole, interfere with the cellular ergosterol synthesis pathway, thereby depleting the cell membrane of its main sterol. Echinocandins, e.g. Caspofungin, inhibit the synthesis of 1,3-β-D-glucan, a mechanism which impairs cell wall formation during growth and budding. Polyenes, e.g. Amphotericin B, disturb the integrity of the fungal membrane directly by pore formation and ion leakage.

For many years, polyenes, especially Amphotericin B, were the only choice in treatment of invasive fungal infections (Terrell 1999). They act fungicidal by disturbing the integrity of the fungal cell membrane (Figure 3) (Odds et al. 2003). Polyenes bind to ergosterol in the

membrane, causing pore formation and ion leakage (Alcazar-Fuoli und Mellado 2014). Polyene drugs can cause serious side effects due to binding to human membrane sterols (Kotler-Brajtburg et al. 1974), therefore triazoles and echinocandins are more common for systemic application nowadays (Odds et al. 2003).

Resistance to antifungal drugs can be defined in two categories (www.eucast.org, (Alcazar-Fuoli und Mellado 2014). Microbiological resistance is defined by the presence of an acquired or intrinsic resistance mechanism in an isolate (Alcazar-Fuoli und Mellado 2014). This state can either be innate (primary resistance) or acquired secondary in response to drug exposure (Alcazar-Fuoli und Mellado 2014). In an antifungal susceptibility test, any isolate is either categorized as susceptible, intermediate or resistant to a certain drug (www.eucast.org). *In vitro* resistance is determined by defined clinical breakpoints and is likely associated with therapeutic failures if the drug choice is not adapted to the resistance pattern.

Resistance can be achieved through different mechanisms. One general mechanism that heightens the tolerance against antifungal agents and can lead to clinical resistance is biofilm formation (Pfaller und Riley 1992). It was observed that yeast cells in biofilms are up to a 1000 times more tolerant against azoles than their planctonic counterparts (Lamfon et al. 2004). This phenomenon is a result of a combination of various single aspects. Biofilm formation is associated with Efflux-pump up-regulation, a changed sterole composition of the membrane and activation of general stress responses (Taff et al. 2013). Additionally, the penetration of antifungal drugs into the biofilm is inhibited by the presence of an extracellular matrix (Mitchell et al. 2013). Irrespective of the presence of a biofilm, cellular stress responses like an elevated cell wall chitin content can generally lead to a resistance to antifungal treatment (Maubon et al. 2014).

Other resistance mechanisms are limited to certain classes of antifungal agents. Resistance against azole drugs can be achieved through three basic mechanisms, which can operate simultaneously by sequential acquisition (Alcazar-Fuoli und Mellado 2014, Maubon et al. 2014). Firstly, an induction of efflux pumps like Cdr1, Cdr2 and Mdr affects all azole drugs by decreasing the intracellular drug concentration (Kanafani und Perfect 2008). Upregulation of the Mdr transporter especially affects fluconazole treatment (Pfaller 2012). The second way to achieve azole resistance is modulation of the target enzyme Erg11p (Li et al. 2004). Azole affinity to the lanosterol-14α-demethylase can be decreased due to multiple point mutations in the *ERG11* gene (Marichal et al. 1999). If the structure of the target enzyme itself is unchanged, a gain-of-function mutation in the transcription factor *UPC2* may lead to a

considerable overexpression of Erg11p, thereby promoting clinical azole resistance (Flowers et al. 2012). Finally, *ERG3* mutations can offer a metabolic bypass to secure membrane integrity by fecosterole synthesis, leading to a reduced effect of azole treatment (Morio et al. 2012).
Reduced susceptibility particularly to echinocandin drugs is mostly caused by mutations in the *FKS* gene, either intrinsic (Barchiesi et al. 2006) or acquired (Beyda et al. 2012).
Resistance mechanisms specifically affecting polyene drugs are rare and mostly associated with mutations in the *ERG3* gene which lead to ergosterole depletion in the membrane (Morio et al. 2012).

Although resistances in *Candida* spp. are still rare (Arendrup 2013), the increasing number of fungal infections and the more excessive use of antifungal drugs make a standardized and reliable antifungal susceptibility testing necessary (Pfaller und Diekema 2012). The Clinical and Laboratory Standard Institute (CLSI) and the EUCAST established standardized antifungal susceptibility testing methods (broth microdilution or commercially available E-test stripes) (Alcazar-Fuoli und Mellado 2014). The ultimate result of these tests is the minimal inhibitory concentration (MIC) of an antifungal agent for each *Candida* spp. isolate (Espinel-Ingroff et al. 2014). Based on the analysis of several MICs from wild-type isolates, the EUCAST committee defined epidemiological cutoff values (ECVs/ECOFFs) (http://www.eucast.org/clinical_breakpoints/). The ECV sets the highest susceptibility endpoint of a wild type-population MIC, thereby assisting the detection of an in vitro resistance (Espinel-Ingroff et al. 2014). While ECVs are argued to be inexpedient, there is a more practical approach combining laboratory and clinical data (Arendrup 2013). CLSI and EUCAST ventured to predict the likelihood of an isolate to be clinically resistant by defining clinical breakpoints (CBP), which correlate with the in vitro MICs (Alcazar-Fuoli und Mellado 2014).

In conclusion, standardized antifungal susceptibility testing provides important information about a set of isolates. Even if the susceptibility testing is not directly related to a clinical treatment recommendation, the data provide valuable information considering cutoff- and breakpoint value setting (Alcazar-Fuoli und Mellado 2014). Standardized testing can be useful in the development of faster and therefore clinically more relevant methods (Alcazar-Fuoli und Mellado 2014). Detection of in vitro resistance is important for better understanding and avoidance of resistance mechanisms (Alcazar-Fuoli und Mellado 2014).

3 Aims of the project

This research project was conducted in the fungal Septomics group of the ZIK Septomics in cooperation with the NRZMyk Jena. In context of the scientific analysis of the pathology of *Candida* bloodstream infections, it is important to take a close look at the variation of clinical isolates.

The main intention of this venture was to provide a genotypically and phenotypically well characterized collection of clinical *Candida albicans* isolates. This strain compilation will be available for further research concerning host-pathogen interaction. It can be utilized to analyze intra-species variations in immune recognition and response triggering in a full-blood model.

Each *Candida albicans* isolate was profiled using MLST, subsequently adding internationally available strain information to the MLST-online database. This part of the project was conducted in cooperation with the current curator of the database, M.-E. Bougnoux (Bougnoux et al. 2002).

Genotype profiles of the *C. albicans* strain collection were phylogenetically reviewed to get insight in the variability of isolates from a geographically limited area. In addition, patient data were surveyed to assess the likelihood of nosocomial transmission of *Candida* strains.

Additionally, the research did offer an insight in the accuracy of species identification in clinical isolates. Awareness of the quality standard of routine, hospital-associated lab work provides a possibility for refinement. Species identification, genotypic characterization and resistance profiling of isolates can be improved by taking advantage of the advanced standards of a reference lab like the NRZMyk.

Another aim of this project was the analysis of the antifungal susceptibility and of the incidence of resistance to antifungal agents in the set of clinical *C. albicans* isolates in cooperation with the NRZMyk. The frequency of resistant strains, combined with patient data such as severity of the illness and clinical outcome, could provide interesting information concerning epidemiology and treatment recommendations (Pfaller 2012).

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Candida albicans bloodstream isolates in a German university hospital are genetically heterogenous and susceptible to commonly used antifungals

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ABSTRACT

From an eight-year-span, 99 *Candida* bloodstream isolates were collected at the University Hospital Wuerzburg, Germany. In this study, all strains were analyzed using molecular and phenotypic typing methods. Confirmatory species identification revealed three isolates that were initially diagnosed as *C. albicans* to be actually *C. dubliniensis*. Two isolates contained a mixed culture of *C. albicans* and *C. glabrata*, in one of the specimens both species could be separated while it was not possible to recover *C. albicans* in the other sample. The remaining 95 *C. albicans* isolates were profiled by multilocus sequence typing (MLST). Phylogenetic analyses showed a highly heterogenous collection of strains, associated with many different clades and constituting a set of new diploid sequence types (DST). For all strains with identical DST, patient data were reviewed for potential nosocomial transmission. In addition, all isolates were tested for their susceptibility to amphotericin B, caspofungin, fluconazole, itraconazole, posaconazole and voriconazole. No clinically relevant resistance could be detected. Furthermore, these data underline that correlation between minimal inhibitory concentrations for caspofungin and anidulafungin is low.

1. Introduction

Candida species are yeasts of the phylum *Ascomycota* and can be isolated from various environmental sources as well as from mammals and humans (McManus and Coleman, 2014). Within the genus, *C. albicans* is the most prevalent and pathogenic of the *Candida* species, causing the majority of systemic and oral candidiasis (Sullivan et al., 2004; Thompson et al., 2010; Zomorodian et al., 2011). In the last decades, *C. albicans* has evolved as a leading cause of hospital acquired infections (Pfaller and Diekema, 2010; Pfaller et al., 2011). In the EPIC-II study, a 1-day point prevalence study involving more then 13,000 patients in 1265 intensive care units, fungi accounted for 19% of all infections and more than 85% of these infections were caused by *Candida* spp. (Vincent et al., 2009). A retrospective analysis of the EPIC-II cohort showed that 12.6% of all positive blood cultures were positive for *Candida* spp. (Kett et al., 2011). Other species in the genus *Candida* can also cause infections and non-*albicans* species generally account for ~50% of hospital acquired *Candida* infections (Perlroth et al., 2007). Although closely related to *C. albicans*, *C. dubliniensis* is a much less virulent pathogen and has initially been associated mainly with oropharyngeal candidiasis in HIV-positive patients (Kurzai et al., 2000; Asmundsdottir et al., 2009; Stokes et al., 2007). *Candida glabrata* on the other hand emerged as a major fungal pathogen during the last decades, mainly due to its resistance against azole antifungals (Rodrigues et al., 2014). In contrast, antifungal drug resistance is generally not a problem in *C. albicans*. Although fluconazole resistant strains have been described, antifungal drug resistance in *C. albicans* remains at very low levels (Alcazar-Fuoli and Mellado, 2014). In a large study, analyzing 256,882 isolates of *Candida* spp. from 41 countries, 98% of *C. albicans* isolates were susceptible to fluconazole (Pfaller et al., 2010).

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1438-4221/

To learn more about the epidemiology and transmission of nosocomial *C. albicans* infections, it is important to characterize single isolates in a distinctive and reproducible way, yielding results that can be shared and compared between different labs. Multilocus sequence typing (MLST) has been shown to fulfill all these requirements (Bougnoux et al., 2002). Single nucleotide polymorphisms in DNA fragments of seven conserved housekeeping genes provide enough discriminatory power to distinguish even closely related isolates. The occurrence of heterozygous positions in the sequenced loci contributes to the individual MLST profile of a *C. albicans* isolate (Bougnoux et al., 2002, 2003; Tavanti et al., 2003). Nowadays, this method is commonly used for molecular typing and epidemiological studies of *C. albicans* (McManus and Coleman, 2014). So far, 18 clades of *C. albicans* were described with one supposed to be more specific for Asia (Odds et al., 2007a; Shin et al., 2011). To date, more than 2000 isolate profiles were collected in the *C. albicans* MLST database (http://calbicans.mlst.net). MLST has proven especially useful for analyzing potential nosocomial transmission of *C. albicans* in a hospital setting (Song et al., 2014). Although most *C. albicans* infections are considered to arise from endogenous infection, horizontal transmission in hospitals has been described (Pfaller, 1996).

Here, we report a MLST- based genotyping of 95 *C. albicans* bloodstream isolates which were collected in a German university hospital. All isolates were tested for antifungal drug resistance. Two *C. albicans* strains were found to be identical and isolated from premature twins, indicating perinatal rather than nosocomial transmission.

2. Material and methods

2.1. Strains and media

Candida bloodstream isolates from the Wuerzburg university hospital were collected over the years 2005–2012 by methods routinely used in diagnostic laboratories. For this study, strains were grown in either liquid or solid yeast extract peptone dextrose (YPD) medium at 37 °C/180 rpm or 35 °C, respectively.

2.2. MLST analysis

According to previous works, we have used seven different loci for the MLST analysis. Therefore, the recommended oligonucleotide primers for the amplification of *AAT1a*, *ACC1*, *ADP1*, *MPI1*, *SYA1*, *VPS13* and *ZWF1b* were used (Bougnoux et al., 2002, 2003; Tavanti et al., 2003). Sequences of these primers are publicly available at the *C. albicans* MLST database homepage (http://calbicans.mlst.net/misc/info.asp).

The PCR was performed on isolated genomic DNA. PCR products were purified using the Invitrogen PureLink Quick PCR Purification Kit (Life Technologies). Purified PCR products were then sequenced by an external company (GATC Biotech, Konstanz, Germany). Both DNA-strands were sequenced and all sequences were reviewed and compared to the MLST database manually. Each sequence chromatogram was scanned in search for heterozygous nucleotide positions, which are characterized by two overlayed, equally strong fluorescence peaks. The MLST profile for each allele was double checked in both forward and reverse sequence. Strain information and diploid sequence types (DSTs) were consequently added to the MLST database (http://calbicans.mlst.net/misc/info.asp).

2.3. Phylogenetic analysis

Phylogenetic analysis was performed based on the MLST information of all strains available in the database. The BURST algorithm was used to cluster the bloodstream isolates into groups and to compare them with the other isolate profiles available in the MLST database (Feil and Enright, 2004; Feil et al., 2004).

2.4. Discrimination of Candida species

Additional tests to secure the species affiliation were conducted on those isolates that posed problems during the MLST analysis. Identification of *C. dubliniensis* included sequencing of the internal transcribed spacer sequence (ITS) of the large ribosomal subunit and failure to form hyphae in liquid RPMI (5 h at 37 °C, 180 rpm). *C. glabrata* and *C. albicans* were identified on CHROMagar Candida after growth for 48 h at 35 °C, in case of *C. glabrata* the ITS region was amplified via PCR and compared to reference strains.

2.5. Antifungal susceptibility testing

To test the susceptibility of the *Candida* bloodstream isolates, we used commercially available E-tests for amphotericin B, caspofungin, fluconazole, itraconazole, posaconazole and voriconazole (Biomerieux). E-tests were applied following the EUCast guidelines, using RPMI agar and 35 °C for incubation. MIC values were read 24 h and 48 h after application. MIC values for fluconazole and anidulafungin were additionally determined using the EUCAST reference methodology. For analysis of the correlation of outcomes for anidulafungin and caspofungin, the MICs for each antifungal drug were plotted using the plotrix package in the statistical environment R. Point size indicates the number of multiple points at the specific position. The dashed regression line was calculated using the lm function and the denoted R-squared coefficient estimates the quality of this linear fit.

3. Results

3.1. MLST-based genotyping of C. albicans bloodstream isolates

The aim of this study was to address population heterogeneity and potential antifungal drug resistance in *C. albicans* bloodstream isolates. For our study we selected 99 *C. albicans* bloodstream isolates that had been stored in the strain collection of the Institute of Hygiene and Microbiology, University of Wuerzburg. All isolates had been retrieved from patients of the University Hospital in Wuerzburg during the years 2005–2012 and identified as *C. albicans*. As an internal control we included three sequential isolates from the same patient (CABI93, -94, and -97). Genotyping of all isolates was performed using multilocus sequence typing (MLST) as described previously (Bougnoux et al., 2003; Tavanti et al., 2003), by determining the diploid sequence types (DST) of defined internal regions of seven housekeeping genes (*AAT1a*, *ACC1*, *ADP1*, *MPIb*, *SYA1*, *VPS13*, and *ZWF1b*). All data were entered into the MLST online database which contains more than 2500 DSTs (http://calbicans.mlst.net). Typing PCRs repeatedly failed to yield amplicons for three isolates (CABI6, CABI11 and CABI49). To confirm their species identity, internal transcribed spacer regions were amplified and sequenced. Based on this, all three isolates could be unequivocally identified as *C. dubliniensis* and were excluded from further analysis. CABI22 was excluded from further analysis as we were unable to retrieve the *C. albicans* isolate from a case of mixed *C. albicans*/*C. glabrata* infection with subcultures repeatedly only growing *C. glabrata*. CABI8, initially identified as *C. albicans*, turned out to be mixed culture of *C. albicans* and *C. glabrata* on CHROMagar. This was confirmed by the sequencing of the ITS region. Consequently, 95 bloodstream *C. albicans* isolates were used in further analyses.

3.2. Analysis of genotyping results

The corresponding sequences of each isolate were assigned using the *C. albicans* MLST database. With 79%, the vast majority of the strains (75 isolates out of 95) displayed an allelic profile that has not been described before, so these new DSTs could be added to the MLST database. A phylogenetic analysis of the bloodstream isolates based on the BURST algorithm revealed that these DSTs were distributed evenly in several groups and clades of *C. albicans*. We did not detect geographical limitations nor restrictions to the clinical origin of the samples (Fig. 1). As shown in Fig. 1, our isolates were either singletons (30%) or distributed over the clonal clusters 1-4 and 9-12 (Fig. 1). The biggest group of 20 isolates clustered around DST 13, which is the most frequently encountered member of *C. albicans* MLST clade 1 (Odds et al., 2007a,b). Clonal clusters 2 (7 isolates, founding DST 155) and 4 (14 isolates, founding DST 124) also shared several bloodstream isolates (Fig. 1).

There was an enrichment of some DSTs within the isolate collection. We found multiple isolates of the sequence types DST 719, DST 1373, DST 2350, DST 2351, DST 2352, DST 2357, and DST 2373 (Table 1). The three sequential isolates of the same patient (CABI93, CABI94 and CABI97) showed an identical diploid sequence type (DST 1373), indicating that there was no additional transmission of *C. albicans* to this patient (Table 1). Furthermore there was one other event where two isolates could be assigned to two patients within a tight time period (CABI32 and CABI39). Both strains had been isolated within 9 days from an identical ward and shared DST2352 (Table 1). Review of patient data was performed to evaluate a potential nosocomial transmission. It turned out that CABI32 and CABI39 had been isolated from two newborn twins in the neonatal medical care unit. Thus, transmission of *C. albicans* from the mother to both twins seems more likely than a postpartal one. A similar case was described at least once in the literature (Doi et al., 1994). For all the other isolates with identical DSTs (Table 1) we did not observe any epidemiological relationship with regard to time of isolation or clinical units.

3.3. Antifungal susceptibility testing

All *Candida* bloodstream isolates were initially tested for their susceptibility against Amphothericin B, caspofungin, fluconazole, itraconazole, posaconazole and voriconazole (using E-test) and fluconazole and anidulafungin (using the EUCAST reference protocol). For Amphothericin B, anidulafungin, fluconazole, posaconazole and voriconazole, no isolate showed MIC values indicating resistance according to EUCAST breakpoints (Fig. 2). For caspofungin, no EUCAST breakpoints have been determined. For itraconazole, the MICs of six isolates as determined by E-test surpassed the EUCAST breakpoint of 0.06 μg/ml (Fig. 2). One isolate, CABI50 had a MIC of 0.5 μg/ml for itraconazole. This isolate was susceptible to the other azoles as well as caspofungin. It displayed however a relative high MIC for amphotericin B with 0.5 μg/ml (Fig. 2). The other five isolates with MICs for itraconazole above the breakpoint were CABI75, CABI80, CABI17, CABI64 and CABI74. None of them showed any significant resistance against the other azoles, caspofungin or amphotericin B (Fig. 2). Therefore, rather than indicating clinical resistance against itraconazole, these data underline that EUCAST breakpoints have to be applied with caution if non-reference methodology is used for determining MICs. To determine the MIC of the bloodstream isolates during the treatment with anidulafungin, we used the EUCAST reference methodology. The EUCAST-defined breakpoint for anidulafungin is 0.03 μg/ml and isolates with a MIC above this value are regarded as resistant (Arendrup et al., 2013). Out of the 95 isolates, only CABI35, CABI64 and CABI66 grew up to a MIC of 0.032 μg/ml (Fig. 3A). In contrast to anidulafungin, no EUCAST breakpoints have yet been defined for caspofungin. As a reason, EUCAST states that there is a high inter-laboratory variation of caspofungin MIC values. To analyze whether a correlation between MIC values for caspofungin and anidulafungin could be observed for the isolates characterized in this study, we plotted MIC values and calculated an R-squared coefficient estimate (Fig. 3B). This analysis clearly indicates that no correlation between the MIC values for both echinocandins can be observed.

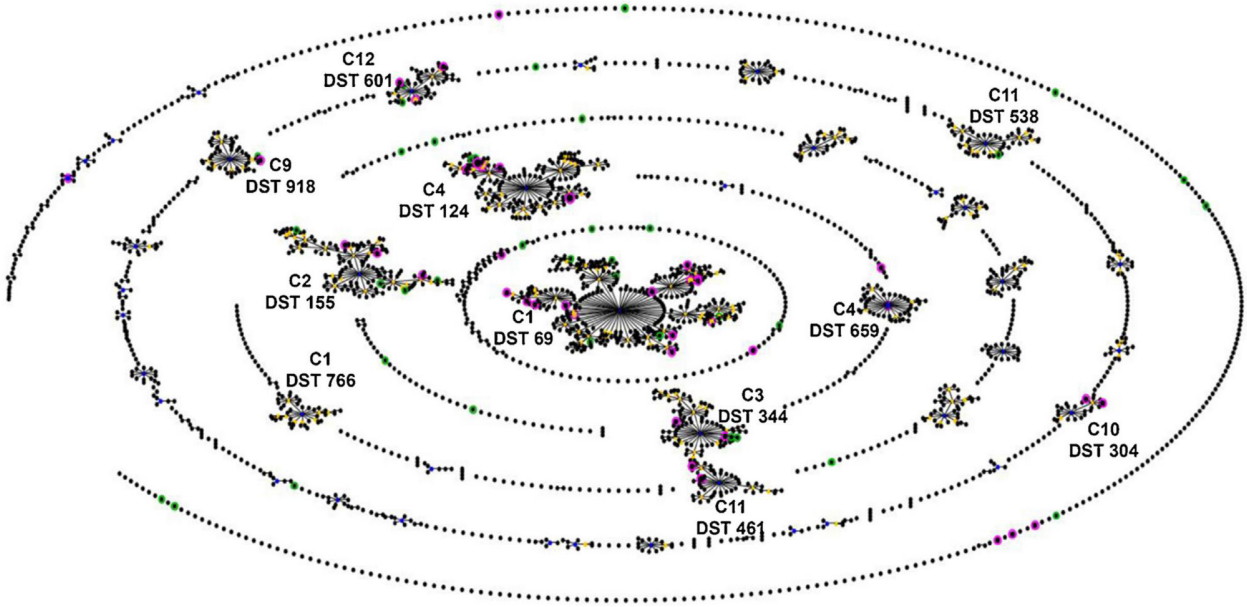

Fig. 1. Distribution of bloodstream isolates among the existing *C. albicans* MLST clades. The eBurst – defined population snapshot is based on all *C. albicans* diploid sequence types (DST) currently available in the *C. albicans* MLST database (www.calbicans.mlst.net). Founding DSTs of clades and groups are shown in blue, founders of subgroups in yellow. *C. albicans* isolates analyzed in this study are shown in green and purple.

Table 1
DST with multiple occurrence in *C. albicans* bloodstream isolates.

DST 719	DST 1373	DST 2350	DST 2351	DST 2352	DST 2357	DST 2373
CABI5	CABI41	CABI3	CABI4	CABI7	CABI14	CABI37
CABI46	CABI59	CABI45	CABI64	CABI32[b]	CABI26	CABI91
CABI76	CABI68	CABI67		CABI39[b]	CABI75	
CABI98	CABI93[a]					
	CABI94[a]					
	CABI97[a]					

No epidemiological relation could be found between the other identical DSTs.
[a] CABI93, CABI94 and CABI97 represent consecutive isolates from a single patient.
[b] CABI32 and CABI39 were isolated within 9 days from twins.

4. Discussion

DNA sequence-based approaches are the state of the art tool for epidemiological studies of *C. albicans* (McManus and Coleman, 2014). Here, we described the molecular typing of bloodstream isolates collected from a German university hospital by applying multilocus sequence typing (MLST, Bougnoux et al., 2002, 2003; Tavanti et al., 2003). It is of interest that three isolates classified as *C. albicans* in initial diagnosis turned out to be *C. dubliniensis*. All cases had been diagnosed before a change of standard operating procedures that made biochemical species confirmation mandatory for bloodstream isolates. Before this, phenotypic identification (green colonies on CHROMagar *Candida*, positive germ-tube test) had been used for identification of *C. albicans*. These tests are known to be unreliable in discriminating *C. albicans* from *C. dubliniensis* (Kurzai et al., 2000). Former reports on the occurrence of *C. dubliniensis* in bloodstream isolates indicate a comparable frequency of *C. dubliniensis* to 3% in Kuwait (Khan et al., 2012) and Scotland (Odds et al., 2007b). The FUNGINOS network recently described a prevalence of 2% for *C. dubliniensis* in *Candida* blood samples isolated in Switzerland (Orasch et al., 2014). In total, the sequence variations of 95 samples were used to define the allelic profile. Most of our samples revealed new diploid sequence types (DST) and 30% of them could not be assigned to any of the existing *C. albicans* clades. Due to the fact that some isolates had the same DST, the overall amount of DSTs was decreased to 86. 20 of these isolates could be assigned to the DST 69-based cluster, which is a well-known member of MLST clade 1, making it the clonal cluster with the most isolates. It was followed by clonal clusters 4 (14 DSTs) and clonal cluster 2 (7 DSTs). Referring to reference strains from the MLST database, clonal cluster 4 represents the MLSt- based *C. albicans* clade 3 and clonal cluster 2 represents clade 4 (Tavanti et al., 2005). Clade 1 is the major sole cluster of our isolates, underlining its importance as the most prevalent clade of *C. albicans* with a global distribution (MacCallum et al., 2009; Ge et al., 2012; Abdulrahim et al., 2013). Two clades are known to be originated from bloodstream isolates: clade 2 which was mostly found in the United Kingdom and clade 4 with origins from the Middle East and Africa (Blignaut et al., 2005; Tavanti et al., 2005; Odds et al., 2007a; Takakura et al., 2008; MacCallum et al., 2009). As all of our

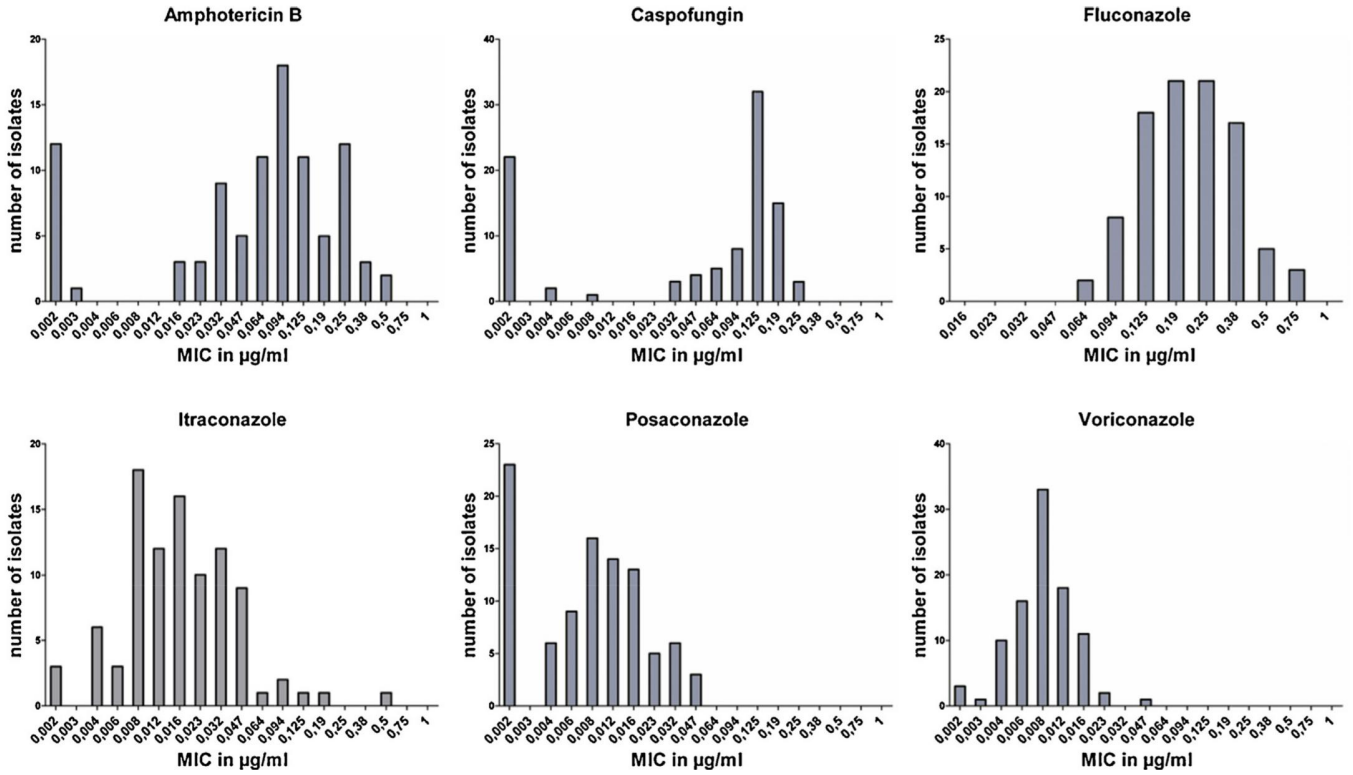

Fig. 2. Distribution of MICs for *C. albicans* bloodstream isolates. All 95 bloodstream isolates were tested for their susceptibility against the indicated antifungal drugs. Fungal cells were plated on RPMI agar and incubated at 35 °C for 48 h after the application of E-test stripes. The minimal inhibitory concentration was determined by EUCAST guidelines for E-tests. Here, the values after the 48 h incubation are shown.

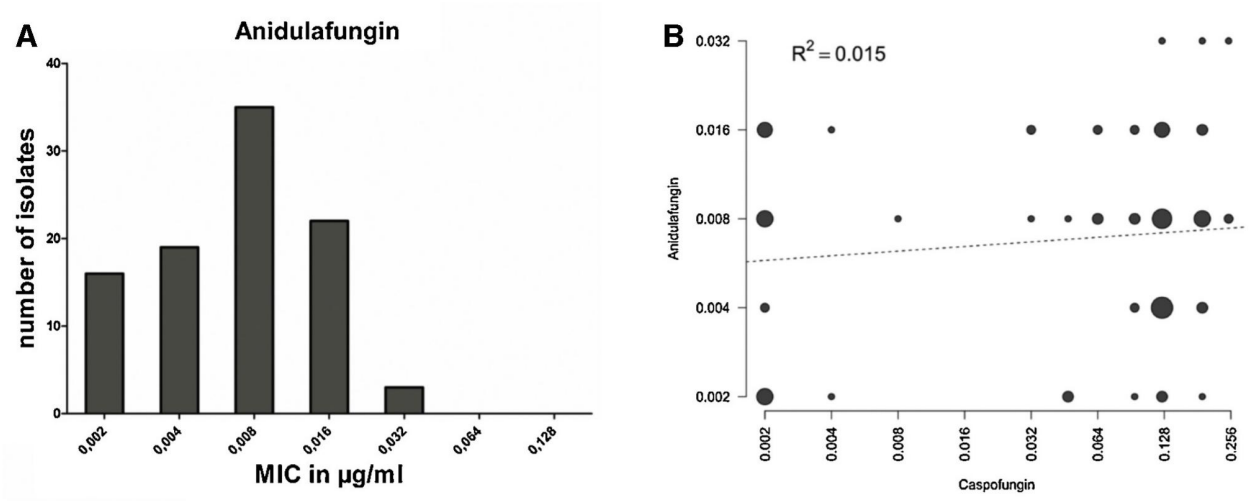

Fig. 3. Susceptibility of *C. albicans* bloodstream isolates to anidulafungin. (A) All 95 bloodstream isolates were tested for their susceptibility against anidulafungin in a microdilution assay. The minimal inhibitory concentration was determined by EUCAST guidelines. Here, the values after the 48 h incubation are shown. (B) The MICs for anidulafungin and caspofungin were plotted against each other. Concentrations are shown in µg/ml for either anidulafungin (*Y* axis) or caspofungin (*X* axis). The larger a single plot is, the more isolates with the same MIC correlation are represented. Dashed line is the regression line, indicating no correlation between the two datasets.

samples were isolated from the bloodstream of patients we would have expected an enrichment of clades 2 and 4, but this was not really the case, especially if we compared the data to clade 1 isolates. As the study was carried out with isolates from Europe, it is not surprising that groups which were more associated with continental Europe in earlier studies (Odds et al., 2007a) are enriched in our samples. None of the *C. albicans* bloodstream isolates belonged to clades 14-18 which seem to be more frequent in Asia (Odds et al., 2007a; Shin et al., 2011). The eBurst clonal cluster 4 (15 DSTs) is highly associated with MLST clade 3, which was originally linked to samples from the USA (Tavanti et al., 2005; Odds et al., 2007a; Takakura et al., 2008). Altogether these data clearly show a large heterogeneity of *C. albicans* bloodstream isolates, which is in line with the endogenous origin of most infections. Most importantly, no evidence for nosocomial transmission could be observed. In one case, an identical DST occurred within a relatively short time period (9 days) on the same ward. Data analysis revealed that the affected patients were premature twins and vertical transmission from the mother seems more likely than horizontal transmission. Although it was not possible to test the mother for fungal infections, cases of this transfer of fungal pathogens from mother to the infants were reported in the past (Doi et al., 1994).

All *C. albicans* bloodstream isolates were susceptible to commonly used antifungal drugs, providing further evidence that in a typical German setting antifungal drug resistance is not an issue with *C. albicans*. Our data clearly indicate that in line with EUCAST recommendations, caution has to be used when applying EUCAST breakpoints to MIC values determined with E-test. Based on the breakpoints published by EUCAST (Arendrup et al., 2013) for itraconazole, six isolates showed MIC values above the threshold using E-test. Based on the results of E-test susceptibility testing for the other azoles as well as testing using the EUCAST reference methodology, we assume that this is a methodological problem rather than clinically relevant resistance. In addition we analyzed susceptibility to echinocandins in all bloodstream isolates. No resistance against anidulafungin could be detected and the MIC distribution reflects reference distributions as published by EUCAST. Interestingly, no correlation could be observed between anidulafungin MICs as determined by the EUCAST reference methodology and caspofungin MICs determined by E-test.

In summary, we provide genotyping and antifungal susceptibility testing data for a set of *C. albicans* bloodstream isolates. Such well characterized collections are essential in further assessing intraspecies variation in virulence and immune recognition of *C. albicans* (Marakalala et al., 2013). Our data clearly show that species identification needs to be constantly refined as early protocols resulted in mis-identification of *C. dubliniensis*. Furthermore, they underline the value of MLST for analyzing clonal relationship between clinical isolates. Indeed, in the first 6 months working period of the NRZMyk in Jena, typing of clinical isolates was requested in several occasions and for a variety of fungal pathogens including *Candida parapsilosis, Aspergillus fumigatus* and *Trichosporon asahii*. The diversity of fungal pathogens and the rare occurrence poses specific challenges and in several cases advanced typing methodologies like MLST are not established. Therefore a national reference lab is clearly required for these analyses which would otherwise be beyond the scope of a routine mycology laboratory. As expected in the setting of this study, nosocomial transmission – although described in the literature – is a rare event for *C. albicans*. Furthermore, in Germany, antifungal drug resistance is typically not an issue in *C. albicans* bloodstream infection for azoles as well as echinocandins, which are considered first-line therapy. A critical use of existing breakpoints is therefore essential to avoid reporting of clinically irrelevant test results.

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References

Abdulrahim, M.H., McManus, B.A., Flint, S.R., Coleman, D.C., 2013. Genotyping *Candida albicans* from *Candida* leukoplakia and non-*Candida* leukoplakia shows no enrichment of multilocus sequence typing clades but enrichment of ABC genotype C in *Candida* leukoplakia. PLoS One 8, e73738.

Alcazar-Fuoli, L., Mellado, E., 2014. Current status of antifungal resistance and its impact on clinical practice. Br. J. Haematol. 166, 471–484.
Arendrup, M.C., Cuenca-Estrella, M., Lass-Florl, C., Hope, W.W., 2013. Breakpoints for antifungal agents: an update from EUCAST focussing on echinocandins against *Candida* spp. and triazoles against *Aspergillus* spp. Drug Resist. Updat. 16, 81–95.
Asmundsdottir, L.R., Erlendsdottir, H., Agnarsson, B.A., Gottfredsson, M., 2009. The importance of strain variation in virulence of *Candida dubliniensis* and *Candida albicans*: results of a blinded histopathological study of invasive candidiasis. Clin. Microbiol. Infect. 15, 576–585.
Blignaut, E., Molepo, J., Pujol, C., Soll, D.R., Pfaller, M.A., 2005. Clade-related amphotericin B resistance among South African *Candida albicans* isolates. Diagn. Microbiol. Infect. Dis. 53, 29–31.
Bougnoux, M.E., Morand, S., d'Enfert, C., 2002. Usefulness of multilocus sequence typing for characterization of clinical isolates of *Candida albicans*. J. Clin. Microbiol. 40, 1290–1297.
Bougnoux, M.E., Tavanti, A., Bouchier, C., Gow, N.A., Magnier, A., Davidson, A.D., Maiden, M.C., D'Enfert, C., Odds, F.C., 2003. Collaborative consensus for optimized multilocus sequence typing of *Candida albicans*. J. Clin. Microbiol. 41, 5265–5266.
Doi, M., Homma, M., Iwaguchi, S., Horibe, K., Tanaka, K., 1994. Strain relatedness of *Candida albicans* strains isolated from children with leukemia and their bedside parents. J. Clin. Microbiol. 32, 2253–2259.
Feil, E.J., Enright, M.C., 2004. Analyses of clonality and the evolution of bacterial pathogens. Curr. Opin. Microbiol. 7, 308–313.
Feil, E.J., Li, B.C., Aanensen, D.M., Hanage, W.P., Spratt, B.G., 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J. Bacteriol. 186, 1518–1530.
Ge, S.H., Xie, J., Xu, J., Li, J., Li, D.M., Zong, L.L., Zheng, Y.C., Bai, F.Y., 2012. Prevalence of specific and phylogenetically closely related genotypes in the population of *Candida albicans* associated with genital candidiasis in China. Fungal Genet. Biol. 49, 86–93.
Kett, D.H., Azoulay, E., Echeverria, P.M., Vincent, J.L., 2011. *Candida* bloodstream infections in intensive care units: analysis of the extended prevalence of infection in intensive care unit study. Crit. Care Med. 39, 665–670.
Khan, Z., Ahmad, S., Joseph, L., Chandy, R., 2012. *Candida dubliniensis*: an appraisal of its clinical significance as a bloodstream pathogen. PLoS One 7, e32952.
Kurzai, O., Korting, H.C., Harmsen, D., Bautsch, W., Molitor, M., Frosch, M., Muhlschlegel, F.A., 2000. Molecular and phenotypic identification of the yeast pathogen *Candida dubliniensis*. J. Mol. Med. (Berl) 78, 521–529.
MacCallum, D.M., Castillo, L., Nather, K., Munro, C.A., Brown, A.J., Gow, N.A., Odds, F.C., 2009. Property differences among the four major *Candida albicans* strain clades. Eukaryot. Cell 8, 373–387.
Marakalala, M.J., Vautier, S., Potrykus, J., Walker, L.A., Shepardson, K.M., Hopke, A., Mora-Montes, H.M., Kerrigan, A., Netea, M.G., Murray, G.I., Maccallum, D.M., Wheeler, R., Munro, C.A., Gow, N.A., Cramer, R.A., Brown, A.J., Brown, G.D., 2013. Differential adaptation of *Candida albicans* in vivo modulates immune recognition by dectin-1. PLoS Pathog. 9, e1003315.
McManus, B.A., Coleman, D.C., 2014. Molecular epidemiology, phylogeny and evolution of *Candida albicans*. Infect. Genet. Evol. 21, 166–178.
Odds, F.C., Bougnoux, M.E., Shaw, D.J., Bain, J.M., Davidson, A.D., Diogo, D., Jacobsen, M.D., Lecomte, M., Li, S.Y., Tavanti, A., Maiden, M.C., Gow, N.A., d'Enfert, C., 2007a. Molecular phylogenetics of *Candida albicans*. Eukaryot. Cell 6, 1041–1052.
Odds, F.C., Hanson, M.F., Davidson, A.D., Jacobsen, M.D., Wright, P., Whyte, J.A., Gow, N.A., Jones, B.L., 2007b. One year prospective survey of *Candida* bloodstream infections in Scotland. J. Med. Microbiol. 56, 1066–1075.
Orasch, C., Marchetti, O., Garbino, J., Schrenzel, J., Zimmerli, S., Muhlethaler, K., Pfyffer, G., Ruef, C., Fehr, J., Zbinden, R., Calandra, T., Bille, J., 2014. *Candida* species distribution and antifungal susceptibility testing according to European Committee on Antimicrobial Susceptibility Testing and new vs. old Clinical and Laboratory Standards Institute clinical breakpoints: a 6-year prospective candidaemia survey from the fungal infection network of Switzerland. Clin. Microbiol. Infect. 20, 698–705.
Perlroth, J., Choi, B., Spellberg, B., 2007. Nosocomial fungal infections: epidemiology, diagnosis, and treatment. Med. Mycol. 45, 321–346.
Pfaller, M.A., 1996. Nosocomial candidiasis: emerging species, reservoirs, and modes of transmission. Clin. Infect. Dis. 22 (Suppl. 2), S89–S94.
Pfaller, M.A., Diekema, D.J., 2010. Epidemiology of invasive mycoses in North America. Crit. Rev. Microbiol. 36, 1–53.
Pfaller, M.A., Diekema, D.J., Gibbs, D.L., Newell, V.A., Ellis, D., Tullio, V., Rodloff, A., Fu, W., Ling, T.A., 2010. Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: a 10.5-year analysis of susceptibilities of *Candida* Species to fluconazole and voriconazole as determined by CLSI standardized disk diffusion. J. Clin. Microbiol. 48, 1366–1377.
Pfaller, M.A., Moet, G.J., Messer, S.A., Jones, R.N., Castanheira, M., 2011. *Candida* bloodstream infections: comparison of species distributions and antifungal resistance patterns in community-onset and nosocomial isolates in the SENTRY Antimicrobial Surveillance Program, 2008–2009. Antimicrob. Agents Chemother. 55, 561–566.
Rodrigues, C.F., Silva, S., Henriques, M., 2014. *Candida glabrata*: a review of its features and resistance. Eur. J. Clin. Microbiol. Infect. Dis. 33, 673–688.
Shin, J.H., Bougnoux, M.E., d'Enfert, C., Kim, S.H., Moon, C.J., Joo, M.Y., Lee, K., Kim, M.N., Lee, H.S., Shin, M.G., Suh, S.P., Ryang, D.W., 2011. Genetic diversity among Korean *Candida albicans* bloodstream isolates: assessment by multilocus sequence typing and restriction endonuclease analysis of genomic DNA by use of BssHII. J. Clin. Microbiol. 49, 2572–2577.
Song, E.S., Shin, J.H., Jang, H.C., Choi, M.J., Kim, S.H., Bougnoux, M.E., d'Enfert, C., Choi, Y.Y., 2014. Multilocus sequence typing for the analysis of clonality among *Candida albicans* strains from a neonatal intensive care unit. Med. Mycol. 52, 653–658.
Stokes, C., Moran, G.P., Spiering, M.J., Cole, G.T., Coleman, D.C., Sullivan, D.J., 2007. Lower filamentation rates of *Candida dubliniensis* contribute to its lower virulence in comparison with *Candida albicans*. Fungal Genet. Biol. 44, 920–931.
Sullivan, D.J., Moran, G.P., Pinjon, E., Al-Mosaid, A., Stokes, C., Vaughan, C., Coleman, D.C., 2004. Comparison of the epidemiology, drug resistance mechanisms, and virulence of *Candida dubliniensis* and *Candida albicans*. FEMS Yeast Res. 4, 369–376.
Takakura, S., Ichiyama, S., Bain, J.M., Davidson, A.D., Jacobsen, M.D., Shaw, D.J., Gow, N.A., Odds, F.C., 2008. Comparison of *Candida albicans* strain types among isolates from three countries. Int. J. Med. Microbiol. 298, 663–668.
Tavanti, A., Davidson, A.D., Fordyce, M.J., Gow, N.A., Maiden, M.C., Odds, F.C., 2005. Population structure and properties of *Candida albicans*, as determined by multilocus sequence typing. J. Clin. Microbiol. 43, 5601–5613.
Tavanti, A., Gow, N.A., Senesi, S., Maiden, M.C., Odds, F.C., 2003. Optimization and validation of multilocus sequence typing for *Candida albicans*. J. Clin. Microbiol. 41, 3765–3776.
Thompson 3rd, G.R., Patel, P.K., Kirkpatrick, W.R., Westbrook, S.D., Berg, D., Erlandsen, J., Redding, S.W., Patterson, T.F., 2010. Oropharyngeal candidiasis in the era of antiretroviral therapy. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 109, 488–495.
Vincent, J.L., Rello, J., Marshall, J., Silva, E., Anzueto, A., Martin, C.D., Moreno, R., Lipman, J., Gomersall, C., Sakr, Y., Reinhart, K., 2009. International study of the prevalence and outcomes of infection in intensive care units. JAMA 302, 2323–2329.
Zomorodian, K., Haghighi, N.N., Rajaee, N., Pakshir, K., Tarazooie, B., Vojdani, M., Sedaghat, F., Vosoghi, M., 2011. Assessment of *Candida* species colonization and denture-related stomatitis in complete denture wearers. Med. Mycol. 49, 208–211.

5 Discussion

The collection of 99 clinical *Candida* isolates could be phylogenetically characterized in depth during the project. It was possible to explicitly determine the species of each isolate: as expected, the major section of the collection consists of *C. albicans* isolates (95 samples). Those strains could be unambiguously defined genotypically using a sequence-based method. Additionally, antifungal susceptibility properties were adjoined to the individual characterization of each strain. The detailed strain delineation that was achieved using the MLST method is essential for the subsequent phylogenetic analysis of the strain compilation and for the review of patient data concerning potential nosocomial transmission. Furthermore, genotypic specification and the following phylogenetic classification of the isolates are necessary groundwork for future research concerning virulence and immune-response variations. Virulence properties depending on phenotyping switching, adhesion, biofilm formation and hydrolytic enzyme production may differ considerable between individual *C. albicans* isolates (Bliss et al. 2012). An enhanced virulence may facilitate the transition from a commensal to an invasive growing isolate (Bliss et al. 2012). Additionally, different invasive isolates of the same species may possibly trigger explicitly different immune reactions under standardized conditions (Toth et al. 2015). Immune recognition may vary between different clinical strains, thereby stimulating a host response of distinct quality and intensity, which will in turn influence growth and phenotype of the pathogen (Marakalala et al. 2013). The dynamics of this host-pathogen-interaction and the influence of genetic micro-variations on the virulence of isolates from a single species are interesting and complex to study. The classifications that were made during this project may contribute a part to this research.

Multilocus sequence typing has proven to be a useful and reliable method for the purposes of this project. In confirmation of the commonly published opinion, we evaluated MLST as an easily conducted, objective and reproducible method with sound and reliable results (Bougnoux et al. 2002). The high quality and reproducibility of this method could be proven during the analysis of the strain collection from Würzburg. The inclusion of three sequential isolates from a single patient served as an internal control of the procedure. Those samples were unequivocally classified as identical, which confirms the eligibility of MLST to analyze clinical isolates. Two other strains with identical DSTs could be retraced to originate from newborn twins, making perinatal transmission from the mother a probable explanation for the congruence. Those results also show that the analyzed DNA fragments are stable enough to

positively identify a strain through the course of infection and transmission, eliminating doubts about the influence of genetic micro-evolution on the certainty of strain recognition (Reiss et al. 1998, Bougnoux et al. 2002). In summary, MLST was estimated as a highly qualified method of distinction for *C. albicans* isolates during this project.

Nosocomial transmission of fungal infections is occasionally mentioned in literature (Pfaller 1996), but cases of horizontal transmission of *C. albicans* infections are seldom and not easily proven. In accordance with this, our review of the patient data belonging to isolates with identical DSTs did not reveal an evident connection between the infected patients. On these grounds, we consider a nosocomial transmission leading to *C. albicans* infections as highly unlikely in question of the analyzed strain collection from Würzburg. The main source for systemic *Candida* infections is endogenous colonization, strains that are maintained by the host over prolonged periods of time before the infection arises (Verduyn Lunel et al. 1999). Nevertheless, there are possibilities to acquire a *Candida* infection from a nosocomial environment, e.g. transfer by health care workers or contaminated biomaterials and medical devices (Pfaller 1996, Verduyn Lunel et al. 1999). In these cases, MLST would be a probate and highly qualified method to identify nosocomial infections and, possibly, the transmission routes (Marcos-Zambrano et al. 2014, Hammarskjold et al. 2013).

In terms of phylogenetic analysis, MLST provides an opportunity to find similarities between different *C. albicans* isolates (Odds et al. 2007). Nowadays, phylogenetic trees can be used to study the evolutionary and epidemiological development of pathogenic species and populations (Yang und Rannala 2012). MLST data can be used to create gene trees, which are built from fragments of homologous genomic DNA from different individuals (Szollosi et al. 2015). The information that this kind of tree is supposed to contain is the evolution of one or more homologous genes – with a theoretical ancestral copy of said gene as a start point, nodes as recombination or mutation events and the branches representing the persistent replication of this sequence copy over time (Yang und Rannala 2012). Therefore, gene trees reflect the genealogy of genomic DNA, which is not equal to the development of a species. In fact, gene trees are more informative if they contain orthologous genes from different species and reach over several specification events (Szollosi et al. 2015). A phylogenetic tree made from MLST data only gained from *C. albicans* bloodstream isolates is a very limited form of a gene tree. It is restricted to homologous sequence fragments of a certain species that were chosen for analysis due to their evolutionary stability. Such a tree is not able to reflect on species diversification or even the development of a gene through duplication, transposition, loss or

horizontal transfer (Szollosi et al. 2015). The only information that is contained in an MLST-gene tree is the variation of polymorphic sites in the sequence, which might occur due to point mutations, insertions or deletions. The existence and recombination of different alleles in diploid species further obscures the evolutionary dynamics. Those kind of trees are hold as highly uncertain, a fact that is supported by the big variety of trees that can be constructed from a single dataset, depending on the algorithm that was used (Yang und Rannala 2012). MLST data are often used to create phylogenetic trees, which are in turn interpreted to separate the contained individuals in multiple clades (Glaeser und Kampfer 2015). This approach might be valuable to get an overview of the genomic variability and relatedness of isolates. But due to the restrictions and incongruence of such a tree it should be kept in mind that it can only give information about the relative distance between single isolates, but is not able to place them definitely at one branch of the tree (respectively in a certain clade) or reflect on its evolution. Besides, the sequence fragments used for an MLST analysis are convenient, but may not be representative for the genome in its entirety (Chaudhuri und Henderson 2012). Summarized, an MLST tree contains, due to sequence choice and the number of isolates, too little information to make a reliable assessment about the evolution of its members. Due to this assessment and multiple failed attempts to produce a congruent tree with the 95 *C. albicans* MLST-profiles, we decided to abstain from offering a definite result of our molecular phylogenetic analysis. Even so, the MLST data provided valuable information concerning the high genetic variability of the isolate collection. As confirmation, an eBURST analysis including all DSTs of the *C. albicans* MLST database was sufficiently able to display the heterogeneous distribution of the analyzed strains among multiple clonal clusters. Against initial expectations, there was no perceptible dominance of certain clades in the isolate collection from the university hospital Würzburg. In a compilation of strains gained only from blood samples and from a geographically very limited area, we would have supposed to discover a grouping of the isolates in few clades or clonal clusters due to their specific characteristics. It was frequently published that characteristics like geographical origin, site of isolation, MAT locus, decade of isolation and susceptibility patterns show noticeable affiliations to the clade number (Odds et al. 2007, Odds und Jacobsen 2008, McManus und Coleman 2014). Interestingly, a significant relation between clade assignment and isolation characteristics could only be proven for geographical traits and mating type (Odds et al. 2007). Since the MAT locus was not analyzed in this study, we concentrated on the geographical enrichments of some clades. For Continental European bloodstream isolates, a clustering of the strains in clade 1, 2, 4, 10 and 11 would have been expected (McManus

und Coleman 2014). Those anticipations were not confirmed in the results of the eBurst diagram or the different dendrograms that were produced during the analysis (results not shown). As previously explained, the delineation of clades from a dendrogram is arbitrary and has no clinical background (Odds und Jacobsen 2008). Due to multiple influence factors and blurring by the international interexchange of host populations, the assumption that clade-associated properties might facilitate the phylogenetic mapping of *C. albicans* isolates should be carefully contemplated. Either the strain compilation of this study is indeed more heterogeneous than expected due to nationality, status and general variability of the patients, which is quite possible in a city with a considerable portion of international students. Or, due to the mentioned infirmities of phylogenetic analysis, a distinct clade mapping is too uncertain to be reliable. Studies with *C. albicans* strains isolated from more homogenous populations in a smaller geographical setting and a more tight time period might help to discover if phylogenetic clade assignments do still have geographical correlations. In terms of specific traits of clades and clonal clusters, it would be desirable to include more metadata from single strains into the MLST database. Currently only new DSTs are added to the data content. Prospectively, it would be useful to add information like anatomical source, geographical origin and susceptibility pattern from single isolates to an already registered DST. This could aid future statistical analysis of the correlation between clade assignment and strain characteristics, thereby either enhancing or disproving the rightfulness of this approach.

Remarkably, five of the isolates that were identified as *C. albicans* at the hospital did not or not exclusively contain a *C. albicans* strain. Three isolates (CABI 6, CABI 11 and CABI 49) were identified as *C. dubliniensis* during the MLST analysis. This misclassification occurs commonly when standard diagnostic procedures, e.g. growth on CHROMagar, Chlamydospore formation or hyphae formation, are used to discriminate *Candida* species (Kurzai et al. 2000). In general, *C. dubliniensis* shares major phenotypic properties with *C. albicans* and was only recognized to be a distinct species in 1995 (Sullivan et al. 1995). *C. dubliniensis´* prevalence, especially in blood samples, was underestimated due to frequent misidentification (Kurzai et al. 2000). To avoid such failures and to accelerate diagnostic procedures, molecular methods like PCR of the species-specific internal transcribed spacer region (ITS) are recommended (Kurzai et al. 2000). To provide a reliable and fast species identification, molecular diagnostic methods should be conventional. Recently, several other quick identification methods have been established for *Candida*. For example, peptide nucleic acid (PNA) – fluorescent in situ hybridization (FISH) and a MALDI-TOF-MS approach (Pfaller und Castanheira 2016). Those tests can be applied directly to blood cultures or agar

cultures and have the great advantage of timeliness and accuracy (results available 1 to 2 hours post culture), which is crucial for an appropriate treatment (Pfaller und Castanheira 2016). Nevertheless, they are expensive and exceed the means of a routine diagnostic lab concerning technology, conduction and practicability.

Our isolate collection was assembled from 2005-2012. Although species identification was no longer solely relied on conventional, phenotype-based methods during that period of time, some misidentifications have occurred. In case of *C. albicans* and *C. dubliniensis*, a confusion of species identity does not have a major impact on the correct and timely application of antifungal therapy. *C. dubliniensis* is in fact considered to be less pathogenic than *C. albicans* (Coleman et al. 2010). Most *C. dubliniensis* isolates are susceptible to antifungal drugs that are commonly used to treat systemic *Candida* infections (Coleman et al. 2010), therefore the misidentifications that we detected in our strain compilation were of no therapeutical consequence at the time of treatment. Contrary, diagnostic errors in the analysis of a blood culture containing *C. glabrata* could deteriorate the prognosis of the patient. The susceptibility patterns of *C. glabrata* are distinctly different than those of *C. albicans* and *C. dubliniensis*: Minimal inhibitory concentrations for most antifungals are higher, acquired resistances are more common and the tolerance against azole antifungals is innately high (Turner und Butler 2014, Pfaller und Castanheira 2016). Therefore, therapeutic failures are more likely in case of a *C. glabrata* infection and an application of echinocandins or polyenes instead of a common triazole therapy is beneficial (Glockner und Cornely 2015, Pfaller und Castanheira 2016). The opportunity to make such a therapeutic decision was lost in one case in our collection of clinical specimens. Among the 99 isolates, we detected two mixed culture containing *C. albicans* as well as *C. glabrata* (CABI 8 and CABI 22). A review of the clinical data revealed that only one was recognized as such during the time of isolation. In case of the other mixed culture, the *C. glabrata* isolate was not identified. However, it can not be excluded that contamination of the original sample occured later during the storage, culture and analysis processes of the strain.

A rapid and sensitive diagnosis of the invasive *Candida* species can ensure the necessity of antifungal treatment and the timely application of the correctly dosed drug. This is an important precondition considering antifungal stewardship and the reduction of therapy costs and the still considerable mortality of Candidemia patients (Pfaller und Castanheira 2016).

Susceptibility to fluconazole, itraconazole, voriconazole, posaconalzole, caspofungin, anidulafungin and amphotericin B was tested for all 95 *C. albicans* isolates following the

EUCAST guidelines. We could successfully estimate the *in vitro* minimal inhibitory concentration (MIC) for each antifungal drug. Using the EUCAST breakpoints, we categorized most isolates as susceptible (six isolates were categorized as resistant to itraconazole, a result which is likely not of clinical relevance and might be owed to methodical difficulties).

This kind of susceptibility testing can identify patients at risk of clinical failure. Nevertheless, microbiological or *in vitro* susceptibility or resistance will not be a reliable predictor of clinical resistance (Maubon et al. 2014). Clinical breakpoints (CBPs) are an attempt to take the clinical outcome, dependent on the virulence of the pathogen and aspects of the host´s health, into account (Maubon et al. 2014). Therefore they provide a slightly better prediction for clinically relevant resistances than the otherwise employed epidemiological cutoff values (ECVs/ECOFFs) (Espinel-Ingroff et al. 2013a). But clinical resistance, defined as a persisting or worsening fungal infection or a breakthrough infection under antifungal therapy, is frequently not caused by a strain with an *in vitro* resistant phenotype. Rather, the general health or sickness of the patient, organ failures, immune suppression and old age play an important role in the failure of antifungal treatment (Maubon et al. 2014). Pharmacodynamic considerations like biofilm formation or infection of sites where antifungal drugs can not penetrate also have an impact (Taff et al. 2013). Additionally, routine susceptibility tests require the growth of purified cultures and are therefore a slow method, while antifungal therapy is a very time-sensitive matter (Pfaller und Castanheira 2016). Overall, the benefit of susceptibility testing for patient-specific treatment recommendations is limited and the results of *in vitro* tests should be carefully interpreted (Maubon et al. 2014).

Nevertheless, regular antifungal susceptibility tests are an indispensable tool to establish valuable antifungal treatment recommendations and to monitor emerging resistances (Alcazar-Fuoli und Mellado 2014, Pfaller und Diekema 2012). Constant monitoring of susceptibilities and the setting and refining of species-specific clinical breakpoints provide valuable assistance in the selection of the most promising first-line antifungal therapy (Alcazar-Fuoli und Mellado 2014). Therefore, antifungal susceptibility testing might not always have a direct, case-specific clinical impact, but should not be neglected due to its epidemiological importance.

Antifungal susceptibility testing is usually conducted using test systems according to the CLSI standards, for example the commercially available Etest (bioMérieux) or microdiluation assays. The tests mostly rely on isolated fungal cultures, although there are efforts to perform

susceptibility tests directly on positive blood cultures in order to accelerate the method (Maubon et al. 2014). The advantage of standardized test systems clearly lies in the opportunity to compare results of different labs considering epidemiological analysis. But next to the fact that they are time-consuming (at least 24 hours before the first results can be estimated), standardized antifungal susceptibility tests are not flawless in their conduction.

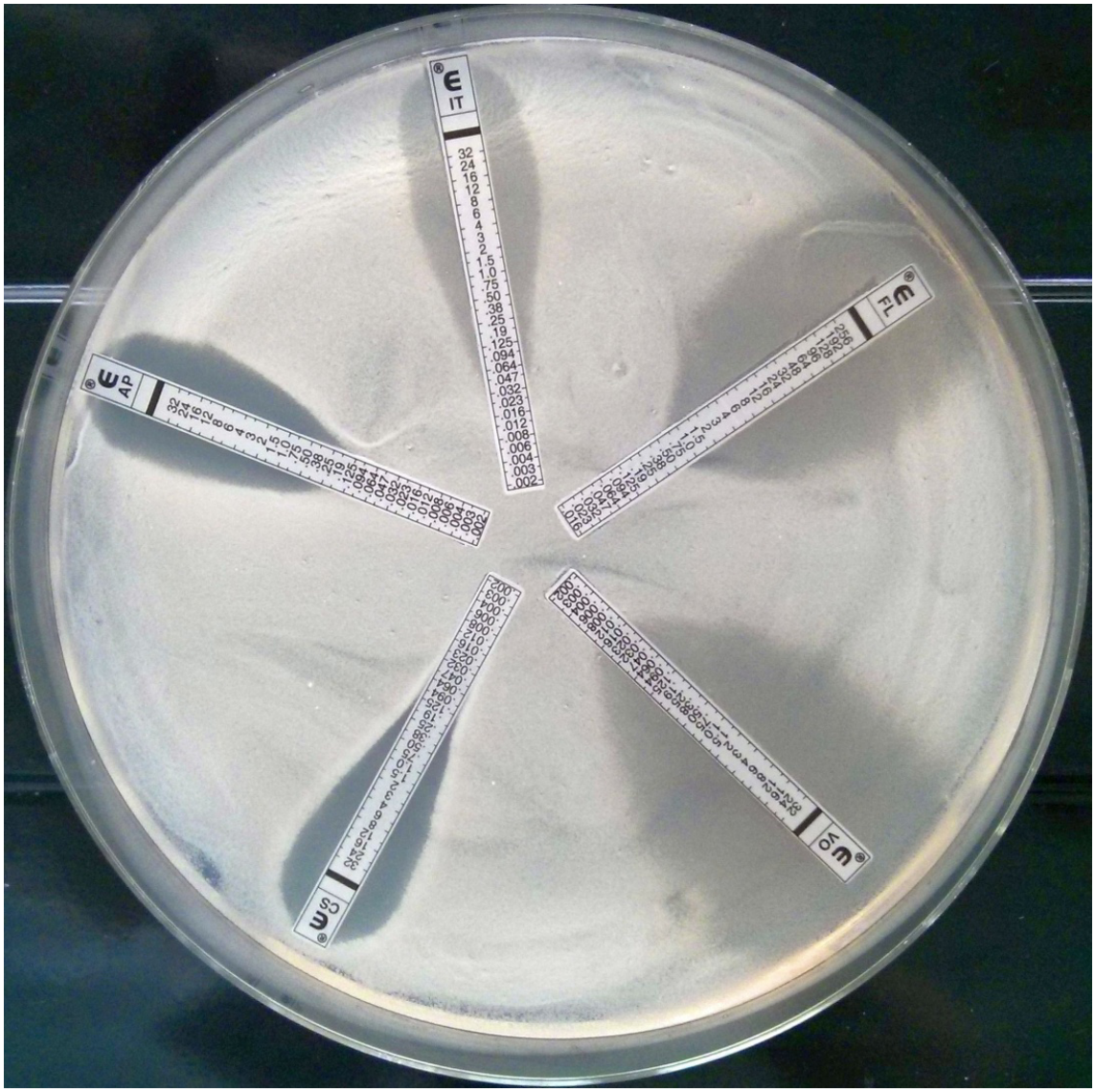

Figure 4: Exemplary Etest from one of the *C. albicans* bloodstream isolates from this project. The image shows the main challenges in Etest conduction and evaluation: firstly, the equal distribution and growth of the exact amount of colony forming units (CFUs). Secondly, the correct reading of the end point of the inhibition zone. For the triazoles, the reading is difficult because of the microcultures in the inhibition zone and the phenomenon of trailing end points. For caspofungin and partly for itraconazole, the slender ending of the bottle-shaped inhibition zone makes a clear reading of the end point challenging.

The accurate performance and especially the interpretation of the results of Etests is not indisputable (Figure 4). Firstly, inoculum size and distribution on the agar plate can be a

source of error. Secondly, the reading of the MIC result requires some experience and is not always unquestionable (Figure 4).

For example, the inhibition zone for triazoles is not colony-free. The occurrence of microcolonies and the phenomenon of trailing end points make the interpretation of the test results difficult (Figure 4). The shape of the inhibition zone itself can also impede the correct reading of the end-point: a drop-shaped inhibition zone is easier to read than a narrow, bottle-shaped one like it is found for itraconazole and caspofungin (Figure 4). This difficulty might explain why six otherwise unobtrusive isolates were classified as resistant against itraconazole according to EUCAST breakpoints. This result might be owed to the difficulties of the method and likely has no clinical impact.

In case of caspofungin, the inter-laboratory variations of antifungal susceptibility testing are significant enough to prevent the EUCAST committee from publishing official break points for interpretation (Espinel-Ingroff et al. 2013b)(www.eucast.org). Alternatively, it is recommended to consider isolates which are susceptible to anidulafungin and micafungin as also susceptible to caspofungin (www.eucast.org). Interestingly, we found no correlation between anidulafungin and caspofungin MICs, rendering a similarity in susceptibility patterns questionable. According to current publications, the lack of correlation might be explained with the uncertainty of caspofungin MIC determination rather than with a significant difference in efficacy (Espinel-Ingroff et al. 2013b). It is controversially disputed if routine *in vitro* testing for caspofungin resistance should be performed at all (Espinel-Ingroff et al. 2013b).

Resistance is still an uncommon trait in *Candida* spp., especially in *C. albicans*. Some species show an intrinsic resistance, for example *C. glabrata* and *C. krusei* have a high tolerance against azoles (Pfaller und Castanheira 2016), while *C. parapsilosis* is less susceptible against echinocandins due to polymorphisms in the *FKS* gene (Maubon et al. 2014). For *C. albicans*, widespread internal resistance mechanisms are highly unusual. But in response to drug exposure, resistance can be acquired through distinct mutations and differential gene expression (Pfaller 2012). Emphasizing the importance of antifungal stewardship, selection pressure through drug exposure is the main reason for the increase of acquired resistances in *Candida* spp. , which was extensively proven for echinocandin and pan-azole resistance in *C. glabrata* (Alexander et al. 2013, Pfaller und Castanheira 2016, Maubon et al. 2014) .
Still, *C. albicans* is considered as unproblematic concerning resistances (Pfaller und Castanheira 2016). Resistance against echinocandins is estimated to occur with a frequency of

less than 1% (Pfaller et al. 2013). Azole resistance can be achieved readily through efflux pump induction (Cdr1, Cdr2 and Mdr1) or mutations of the target enzyme (ERG11p) and other enzymes of the sterole metabolism (ERG3p) (Li et al. 2004). But overall, with merely 0.4% azole resistance, *C. albicans* is not a source for major concerns yet (Pfaller et al. 2013). Polyene, respectively Amphotericin B *in vitro* resistance, is very rare in all *Candida* species and therefore has no significant impact (Maubon et al. 2014).
Overall, the results of the antifungal susceptibility tests conducted in this project matched the prior expectations. No significant discrepancies or alarming resistance patterns were found. According to the EUCAST clinical breakpoints, all *C. albicans* isolates were susceptible to almost all commonly used antifungal drugs, with attention to the major host and pharmacodynamic facts an ideal condition for a favorable clinical outcome.

Despite these inoffensive results, a close examination of the usefulness and advantages of antifungal susceptibility testing showed the necessity of routine testing and scanning for emerging resistances as a main pillar of antifungal stewardship. Next to the limited and considerate use of antifungal drugs, susceptibility testing can help to prevent a problematic increase of resistances, especially considering non-*albicans Candida* species.

6 Conclusions

In the years between 2005 and 2012, 99 *Candida* spp. isolates were gathered from the bloodstream of patients in the university hospital Würzburg. Initially, all isolates were presumed to be *C. albicans* specimens. Retrospective species identification revealed that three isolates contained *C. dubliniensis* and two specimens were mixed cultures of *C. glabrata* and *C. albicans*. Possible sources and consequences of misidentification and the importance of an accurate and timely identification of *Candida* bloodstream infections on species level were discussed previously in the thesis, but are not the major aim of this project.

All 95 *C. albicans* isolates were molecularly analyzed with the MLST method. Typing results were subsequently added to the MLST online database. The majority of the isolates (79%) featured an allelic profile that was not previously described and consequently added as a new DST to the database. Seven DSTs were assigned to more than one isolate, thereupon the patient data from these isolates were reviewed concerning the possibility of nosocomial transmission. This investigation produced no sign of a patient-to-patient strain transmission, a result that was expected due to the fact that proven cases of nosocomial transmission of *C. albicans* were not published yet. Our results showed unambiguously that MLST is an appropriate tool to investigate nosocomial transmission and the progression of invasive infections. A phylogenetic analysis of the MLST data with the eBurst algorithm distributed the isolates widely in several clonal clusters, hinting that the strains are genetically heterogeneous and not closely related. In résumé, MLST is a well standardized and reliable method suitable for epidemiological and phylogenetic analysis and the detailed characterization of *C. albicans* isolates.

Finally, all *C. albicans* isolates were tested for their susceptibility against common antifungal drugs. According to CLSI and EUCAST recommendations, Etests and a microdilution method were used to conduct the analysis. Except for some insignificant aberrations in case of itraconazole, no unusual tolerances against antifungal medication were found in the 95 isolates. Considering the sparse frequency of innate or acquired resistance in *C. albicans*, this result was not unexpected. Interestingly, we found no correlation between anidulafungin and caspofungin MICs, a fact that renders the commonly published recommendation to equalize anidulafungin or micafungin susceptibility with caspofungin susceptibility questionable. Although antifungal susceptibility test are, as previously discussed, not ideal for patient-specific candidemia treatment recommendations, they are an important epidemiological tool

for the development of general treatment recommendations and the surveillance of emerging resistances.

7 References

Alcazar-Fuoli L, Mellado E. 2014. Current status of antifungal resistance and its impact on clinical practice. Br J Haematol, 166 (4):471-484.

Alexander BD, Johnson MD, Pfeiffer CD, Jimenez-Ortigosa C, Catania J, Booker R, Castanheira M, Messer SA, Perlin DS, Pfaller MA. 2013. Increasing echinocandin resistance in Candida glabrata: clinical failure correlates with presence of FKS mutations and elevated minimum inhibitory concentrations. Clin Infect Dis, 56 (12):1724-1732.

Arendrup MC. 2013. Candida and candidaemia. Susceptibility and epidemiology. Dan Med J, 60 (11):B4698.

Barchiesi F, Spreghini E, Tomassetti S, Della Vittoria A, Arzeni D, Manso E, Scalise G. 2006. Effects of caspofungin against Candida guilliermondii and Candida parapsilosis. Antimicrob Agents Chemother, 50 (8):2719-2727.

Bassetti M, Taramasso L, Nicco E, Molinari MP, Mussap M, Viscoli C. 2011. Epidemiology, species distribution, antifungal susceptibility and outcome of nosocomial candidemia in a tertiary care hospital in Italy. PLoS One, 6 (9):e24198.

Bennett RJ, Johnson AD. 2003. Completion of a parasexual cycle in Candida albicans by induced chromosome loss in tetraploid strains. EMBO J, 22 (10):2505-2515.

Beyda ND, Lewis RE, Garey KW. 2012. Echinocandin resistance in Candida species: mechanisms of reduced susceptibility and therapeutic approaches. Ann Pharmacother, 46 (7-8):1086-1096.

Bliss JM, Wong AY, Bhak G, Laforce-Nesbitt SS, Taylor S, Tan S, Stoll BJ, Higgins RD, Shankaran S, Benjamin DK, Jr., Candida Subcommittee of the Eunice Kennedy Shriver National Institute of Child H, Human Development Neonatal Research N. 2012. Candida virulence properties and adverse clinical outcomes in neonatal candidiasis. J Pediatr, 161 (3):441-447 e442.

Bougnoux ME, Morand S, d'Enfert C. 2002. Usefulness of multilocus sequence typing for characterization of clinical isolates of Candida albicans. J Clin Microbiol, 40 (4):1290-1297.

Bougnoux ME, Aanensen DM, Morand S, Theraud M, Spratt BG, d'Enfert C. 2004. Multilocus sequence typing of Candida albicans: strategies, data exchange and applications. Infect Genet Evol, 4 (3):243-252.

Bougnoux ME, Kac G, Aegerter P, d'Enfert C, Fagon JY, CandiRea Study G. 2008. Candidemia and candiduria in critically ill patients admitted to intensive care units in France: incidence, molecular diversity, management and outcome. Intensive Care Med, 34 (2):292-299.

Bougnoux ME, Tavanti A, Bouchier C, Gow NA, Magnier A, Davidson AD, Maiden MC, D'Enfert C, Odds FC. 2003. Collaborative consensus for optimized multilocus sequence typing of Candida albicans. J Clin Microbiol, 41 (11):5265-5266.

Bougnoux ME, Diogo D, Francois N, Sendid B, Veirmeire S, Colombel JF, Bouchier C, Van Kruiningen H, d'Enfert C, Poulain D. 2006. Multilocus sequence typing reveals intrafamilial transmission and microevolutions of Candida albicans isolates from the human digestive tract. J Clin Microbiol, 44 (5):1810-1820.

Chaudhuri RR, Henderson IR. 2012. The evolution of the Escherichia coli phylogeny. Infect Genet Evol, 12 (2):214-226.

Chibana H, Beckerman JL, Magee PT. 2000. Fine-resolution physical mapping of genomic diversity in Candida albicans. Genome Res, 10 (12):1865-1877.

Coleman DC, Moran GP, McManus BA, Sullivan DJ. 2010. Mechanisms of antifungal drug resistance in Candida dubliniensis. Future Microbiol, 5 (6):935-949.

Colombo AL, Guimaraes T, Sukienik T, Pasqualotto AC, Andreotti R, Queiroz-Telles F, Nouer SA, Nucci M. 2014. Prognostic factors and historical trends in the epidemiology of candidemia in critically ill patients: an analysis of five multicenter studies sequentially conducted over a 9-year period. Intensive Care Med, 40 (10):1489-1498.

Espinel-Ingroff A, Cuenca-Estrella M, Canton E. 2013a. EUCAST and CLSI: working together towards a harmonized method for antifungal susceptibility testing.

Espinel-Ingroff A, Pfaller MA, Bustamante B, Canton E, Fothergill A, Fuller J, Gonzalez GM, Lass-Florl C, Lockhart SR, Martin-Mazuelos E, Meis JF, Melhem MS, Ostrosky-Zeichner L, Pelaez T, Szeszs MW, St-Germain G, Bonfietti LX, Guarro J, Turnidge J. 2014. Multilaboratory study of epidemiological cutoff values for detection of resistance in eight Candida species to

fluconazole, posaconazole, and voriconazole. Antimicrob Agents Chemother, 58 (4):2006-2012.
Espinel-Ingroff A, Arendrup MC, Pfaller MA, Bonfietti LX, Bustamante B, Canton E, Chryssanthou E, Cuenca-Estrella M, Dannaoui E, Fothergill A, Fuller J, Gaustad P, Gonzalez GM, Guarro J, Lass-Florl C, Lockhart SR, Meis JF, Moore CB, Ostrosky-Zeichner L, Pelaez T, Pukinskas SR, St-Germain G, Szeszs MW, Turnidge J. 2013b. Interlaboratory variability of Caspofungin MICs for Candida spp. Using CLSI and EUCAST methods: should the clinical laboratory be testing this agent? Antimicrob Agents Chemother, 57 (12):5836-5842.
Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol, 186 (5):1518-1530.
Flowers SA, Barker KS, Berkow EL, Toner G, Chadwick SG, Gygax SE, Morschhauser J, Rogers PD. 2012. Gain-of-function mutations in UPC2 are a frequent cause of ERG11 upregulation in azole-resistant clinical isolates of Candida albicans. Eukaryot Cell, 11 (10):1289-1299.
Forche A, Magee PT, Selmecki A, Berman J, May G. 2009. Evolution in Candida albicans populations during a single passage through a mouse host. Genetics, 182 (3):799-811.
Giri S, Kindo AJ. 2012. A review of Candida species causing blood stream infection. Indian J Med Microbiol, 30 (3):270-278.
Glaeser SP, Kampfer P. 2015. Multilocus sequence analysis (MLSA) in prokaryotic taxonomy. Syst Appl Microbiol, 38 (4):237-245.
Glockner A, Cornely OA. 2015. Candida glabrata--unique features and challenges in the clinical management of invasive infections. Mycoses, 58 (8):445-450.
Gudlaugsson O, Gillespie S, Lee K, Vande Berg J, Hu J, Messer S, Herwaldt L, Pfaller M, Diekema D. 2003. Attributable mortality of nosocomial candidemia, revisited. Clin Infect Dis, 37 (9):1172-1177.
Hall BG. 2013. Building phylogenetic trees from molecular data with MEGA. Mol Biol Evol, 30 (5):1229-1235.
Hammarskjold F, Mernelius S, Andersson RE, Berg S, Hanberger H, Lofgren S, Malmvall BE, Petzold M, Matussek A. 2013. Possible transmission of Candida albicans on an intensive care unit: genotype and temporal cluster analyses. J Hosp Infect, 85 (1):60-65.
Kanafani ZA, Perfect JR. 2008. Antimicrobial resistance: resistance to antifungal agents: mechanisms and clinical impact. Clin Infect Dis, 46 (1):120-128.
Kett DH, Azoulay E, Echeverria PM, Vincent JL, Extended Prevalence of Infection in ICUSGoI. 2011. Candida bloodstream infections in intensive care units: analysis of the extended prevalence of infection in intensive care unit study. Crit Care Med, 39 (4):665-670.
Kotler-Brajtburg J, Price HD, Medoff G, Schlessinger D, Kobayashi GS. 1974. Molecular basis for the selective toxicity of amphotericin B for yeast and filipin for animal cells. Antimicrob Agents Chemother, 5 (4):377-382.
Kumamoto CA, Vinces MD. 2005. Alternative Candida albicans lifestyles: growth on surfaces. Annu Rev Microbiol, 59:113-133.
Kurzai O, Korting HC, Harmsen D, Bautsch W, Molitor M, Frosch M, Muhlschlegel FA. 2000. Molecular and phenotypic identification of the yeast pathogen Candida dubliniensis. J Mol Med (Berl), 78 (9):521-529.
Lamfon H, Porter SR, McCullough M, Pratten J. 2004. Susceptibility of Candida albicans biofilms grown in a constant depth film fermentor to chlorhexidine, fluconazole and miconazole: a longitudinal study. J Antimicrob Chemother, 53 (2):383-385.
Li X, Brown N, Chau AS, Lopez-Ribot JL, Ruesga MT, Quindos G, Mendrick CA, Hare RS, Loebenberg D, DiDomenico B, McNicholas PM. 2004. Changes in susceptibility to posaconazole in clinical isolates of Candida albicans. J Antimicrob Chemother, 53 (1):74-80.
Lockhart SR, Iqbal N, Cleveland AA, Farley MM, Harrison LH, Bolden CB, Baughman W, Stein B, Hollick R, Park BJ, Chiller T. 2012. Species identification and antifungal susceptibility testing of Candida bloodstream isolates from population-based surveillance studies in two U.S. cities from 2008 to 2011. J Clin Microbiol, 50 (11):3435-3442.
Lyon JP, Moraes KC, Moreira LM, Aimbire F, de Resende MA. 2010. Candida albicans: genotyping methods and clade related phenotypic characteristics. Braz J Microbiol, 41 (4):841-849.

Marakalala MJ, Vautier S, Potrykus J, Walker LA, Shepardson KM, Hopke A, Mora-Montes HM, Kerrigan A, Netea MG, Murray GI, Maccallum DM, Wheeler R, Munro CA, Gow NA, Cramer RA, Brown AJ, Brown GD. 2013. Differential adaptation of Candida albicans in vivo modulates immune recognition by dectin-1. PLoS Pathog, 9 (4):e1003315.

Marcos-Zambrano LJ, Escribano P, Bouza E, Guinea J. 2014. [Use of molecular typing tools for the study of hospital outbreaks of candidemia]. Rev Iberoam Micol, 31 (2):97-103.

Marichal P, Koymans L, Willemsens S, Bellens D, Verhasselt P, Luyten W, Borgers M, Ramaekers FC, Odds FC, Bossche HV. 1999. Contribution of mutations in the cytochrome P450 14alpha-demethylase (Erg11p, Cyp51p) to azole resistance in Candida albicans. Microbiology, 145 (Pt 10):2701-2713.

Maubon D, Garnaud C, Calandra T, Sanglard D, Cornet M. 2014. Resistance of Candida spp. to antifungal drugs in the ICU: where are we now? Intensive Care Med, 40 (9):1241-1255.

McManus BA, Coleman DC. 2014. Molecular epidemiology, phylogeny and evolution of Candida albicans. Infect Genet Evol, 21:166-178.

Mikulska M, Del Bono V, Ratto S, Viscoli C. 2012. Occurrence, presentation and treatment of candidemia. Expert Rev Clin Immunol, 8 (8):755-765.

Mitchell KF, Taff HT, Cuevas MA, Reinicke EL, Sanchez H, Andes DR. 2013. Role of matrix beta-1,3 glucan in antifungal resistance of non-albicans Candida biofilms. Antimicrob Agents Chemother, 57 (4):1918-1920.

Moran G, Stokes C, Thewes S, Hube B, Coleman DC, Sullivan D. 2004. Comparative genomics using Candida albicans DNA microarrays reveals absence and divergence of virulence-associated genes in Candida dubliniensis. Microbiology, 150 (Pt 10):3363-3382.

Morio F, Pagniez F, Lacroix C, Miegeville M, Le Pape P. 2012. Amino acid substitutions in the Candida albicans sterol Delta5,6-desaturase (Erg3p) confer azole resistance: characterization of two novel mutants with impaired virulence. J Antimicrob Chemother, 67 (9):2131-2138.

Mukherjee PK, Sendid B, Hoarau G, Colombel JF, Poulain D, Ghannoum MA. 2015. Mycobiota in gastrointestinal diseases. Nat Rev Gastroenterol Hepatol, 12 (2):77-87.

Nei M, Kumar S. 2000. Molecular evolution and phylogenetics New York: Oxford University Press.

Odds FC, Jacobsen MD. 2008. Multilocus sequence typing of pathogenic Candida species. Eukaryot Cell, 7 (7):1075-1084.

Odds FC, Brown AJ, Gow NA. 2003. Antifungal agents: mechanisms of action. Trends Microbiol, 11 (6):272-279.

Odds FC, Bougnoux ME, Shaw DJ, Bain JM, Davidson AD, Diogo D, Jacobsen MD, Lecomte M, Li SY, Tavanti A, Maiden MC, Gow NA, d'Enfert C. 2007. Molecular phylogenetics of Candida albicans. Eukaryot Cell, 6 (6):1041-1052.

Pfaller M, Riley J. 1992. Effects of fluconazole on the sterol and carbohydrate composition of four species of Candida. Eur J Clin Microbiol Infect Dis, 11 (2):152-156.

Pfaller MA. 1996. Nosocomial candidiasis: emerging species, reservoirs, and modes of transmission. Clin Infect Dis, 22 Suppl 2:S89-94.

Pfaller MA. 2012. Antifungal drug resistance: mechanisms, epidemiology, and consequences for treatment. Am J Med, 125 (1 Suppl):S3-13.

Pfaller MA, Diekema DJ. 2012. Progress in antifungal susceptibility testing of Candida spp. by use of Clinical and Laboratory Standards Institute broth microdilution methods, 2010 to 2012. J Clin Microbiol, 50 (9):2846-2856.

Pfaller MA, Castanheira M. 2016. Nosocomial Candidiasis: Antifungal Stewardship and the Importance of Rapid Diagnosis. Med Mycol, 54 (1):1-22.

Pfaller MA, Messer SA, Woosley LN, Jones RN, Castanheira M. 2013. Echinocandin and triazole antifungal susceptibility profiles for clinical opportunistic yeast and mold isolates collected from 2010 to 2011: application of new CLSI clinical breakpoints and epidemiological cutoff values for characterization of geographic and temporal trends of antifungal resistance. J Clin Microbiol, 51 (8):2571-2581.

Planet PJ. 2006. Tree disagreement: measuring and testing incongruence in phylogenies. J Biomed Inform, 39 (1):86-102.

Pujol C, Pfaller MA, Soll DR. 2004. Flucytosine resistance is restricted to a single genetic clade of Candida albicans. Antimicrob Agents Chemother, 48 (1):262-266.

Rajilic-Stojanovic M, de Vos WM. 2014. The first 1000 cultured species of the human gastrointestinal microbiota. FEMS Microbiol Rev, 38 (5):996-1047.
Reiss E, Tanaka K, Bruker G, Chazalet V, Coleman D, Debeaupuis JP, Hanazawa R, Latge JP, Lortholary J, Makimura K, Morrison CJ, Murayama SY, Naoe S, Paris S, Sarfati J, Shibuya K, Sullivan D, Uchida K, Yamaguchi H. 1998. Molecular diagnosis and epidemiology of fungal infections. Med Mycol, 36 Suppl 1:249-257.
Robles JC, Koreen L, Park S, Perlin DS. 2004. Multilocus sequence typing is a reliable alternative method to DNA fingerprinting for discriminating among strains of Candida albicans. J Clin Microbiol, 42 (6):2480-2488.
Saghrouni F, Ben Abdeljelil J, Boukadida J, Ben Said M. 2013. Molecular methods for strain typing of Candida albicans: a review. J Appl Microbiol, 114 (6):1559-1574.
Spratt BG, Hanage WP, Li B, Aanensen DM, Feil EJ. 2004. Displaying the relatedness among isolates of bacterial species -- the eBURST approach. FEMS Microbiol Lett, 241 (2):129-134.
Stokes C, Moran GP, Spiering MJ, Cole GT, Coleman DC, Sullivan DJ. 2007. Lower filamentation rates of Candida dubliniensis contribute to its lower virulence in comparison with Candida albicans. Fungal Genet Biol, 44 (9):920-931.
Sullivan DJ, Westerneng TJ, Haynes KA, Bennett DE, Coleman DC. 1995. Candida dubliniensis sp. nov.: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. Microbiology, 141 (Pt 7):1507-1521.
Szollosi GJ, Tannier E, Daubin V, Boussau B. 2015. The inference of gene trees with species trees. Syst Biol, 64 (1):e42-62.
Taff HT, Mitchell KF, Edward JA, Andes DR. 2013. Mechanisms of Candida biofilm drug resistance. Future Microbiol, 8 (10):1325-1337.
Tavanti A, Gow NA, Senesi S, Maiden MC, Odds FC. 2003. Optimization and validation of multilocus sequence typing for Candida albicans. J Clin Microbiol, 41 (8):3765-3776.
Terrell CL. 1999. Antifungal agents. Part II. The azoles. Mayo Clin Proc, 74 (1):78-100.
Toth R, Alonso MF, Bain JM, Vagvolgyi C, Erwig LP, Gacser A. 2015. Different Candida parapsilosis clinical isolates and lipase deficient strain trigger an altered cellular immune response. Front Microbiol, 6:1102.
Turner SA, Butler G. 2014. The Candida pathogenic species complex. Cold Spring Harb Perspect Med, 4 (9):a019778.
Verduyn Lunel FM, Meis JF, Voss A. 1999. Nosocomial fungal infections: candidemia. Diagn Microbiol Infect Dis, 34 (3):213-220.
Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB. 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin Infect Dis, 39 (3):309-317.
Yang ZH, Rannala B. 2012. Molecular phylogenetics: principles and practice. Nature Reviews Genetics, 13 (5):303-314.

8 Appendix

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8.3 Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: Ronny Martin, Oliver Kurzai.

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen
von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht
bei einer anderen Hochschule als Dissertation eingereicht habe.

Ort, Datum Unterschrift des Verfassers